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Author	Rafferty, Teresa S.
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THE EFFECT OF SELENIUM ON ULTRAVIOLET-B RADIATION-INDUCED DAMAGE TO THE SKIN

Thesis submitted for the Degree of Doctor of Philosophy by Teresa S. Rafferty

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I dedicate this thesis to my mother and father and sister Karen for their never ending support and to Derek for always being there.

Declaration.

I declare that the studies presented here are the result of my own independent investigation, with the exceptions of the measurements of selenium content of the tissue culture media, plasma and skin of patients which were carried out by Professor J Arthur at the Rowett Research Institute, Aberdeen.

This work has not been submitted for candidature for any other degree.

Teresa S. Rafferty

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Abstract

Ultraviolet (UV) irradiation causes non-melanoma skin cancers, the incidence of which have dramatically increased. UV irradiation can damage the skin in many ways, it induces DNA damage, inflammation, immune suppression, oxidative damage and can lead to cell death.

Selenium (Se) is a trace element found in many food sources, it is incorporated into specific selenoproteins. These include glutathione peroxidase and thioredoxin reductase which have strong antioxidant functions. In mice Se supplementation can reduce the number of skin tumours formed after UV irradiation. The aim of this thesis was to characterise how Se can act in the skin to prevent ultraviolet- B radiation (UVB)-induced cellular damage.

To assess the effect of Se supplementation on UV irradiation of the skin, human skin cells were grown *in vitro* and supplemented with Se. Selenoprotein profiles were characterised using [^{75}Se]-labelling. It was shown that primary human fibroblasts, melanocytes and keratinocytes express unique patterns of selenoproteins. Se was found to diminish the amount of cell death induced by UVB, Se also decreased the level of UVB-induced apoptotic cell death. Following UVB irradiation keratinocytes produce cytokines. In primary human keratinocytes Se diminished the induction of mRNA for IL-8, IL-6 but not the protein levels of these cytokines. However Se supplementation did decrease both the mRNA and protein levels of human TNF-α. In mouse keratinocytes the UVB-induction of the mRNA for TNF-α and IL-10 were decreased as were the IL-10 protein levels.

The effect of Se on the UVB-induced DNA damage was assessed by the comet assay. The formation and rate of repair of cyclobutane dimer sites was not affected by Se. However the formation of oxidative DNA damage was decreased. The p53 protein is also induced following UVB irradiation of keratinocytes, Se did not affect this induction. Se was also found to decrease the production of lipid peroxides in keratinocytes following exposure to UVB. Finally mice were fed diets containing different levels of Se for six

weeks which resulted in increased GPX activity in Lymph nodes, skin and livers. The mice were then exposed to UVB. It was found that the number of epidermal Langerhans' cells were higher in Se replete animals and remained higher even after UVB treatment.

Overall it would appear that Se supplementation can decrease many forms of UVB-induced damage in the skin.

List of Abbreviations

Activator protein-1	AP-1
Adenosine diphosphate	ADP
Adenosine triphosphate	ATP
Analysis of variance	ANOVA
Antigen presenting cell	APC
Arabinosyl cytosine	ARAC
α-tocopherol	Vitamin E
Basal cell carcinoma	BCC
B cell leukaemia-2	Bcl-2
Bovine serum albumin	BSA
Carbon dioxide	CO_2
Catalase	CAT
Cell death gene	CED
Cluster of differentiation	CD
Colony stimulating factor	CSF
Complimentary DNA	cDNA
Cyclin dependent kinase	CDK
Cyclopyrimidine dimers	CPDs
Cytoplasmic glutathione peroxidase	cGPX
Deoxyribonucleic acid	DNA
Dinucleotide triphosphates	dNTPs
Dithiolthreitol	DTT
Dulbeccos' modified eagles media	DMEM
Earles' balanced salt solution	EBSS
Enhanced chemiluminescence	ECL
Enzyme-linked immunosorbent assay	ELISA
Extracelluar glutathione peroxidase	EGPX
Fas-associated protein with death domain	FADD
Foetal calf serum	FCS
Foramidopyrimidine-DNA glycosylase	FaPy-glycosylase
Gamma-interferon	γ-INF
Glutathione	GSH
Glutathione peroxidase	GPX
Glutathione reductase	GR

Horse radish peroxidase	HRP
Hydrochloric acid	HCl
Hydrogen peroxide	H_20_2
8-hydroxyguanosine	8-OHdg
Inducible nitric oxide synthetase	iNOS
Interleukin	IL
Intracellular adhesion molecule-1	ICAM-1
Iodothyronine 5-deiodinases	IDI
Joules/metre^2	J/m^2
Keratinocytes serum-free media	K-SFM
Langerhans' cells	LCs
Lipid peroxides	LOOH
Magnesium chloride	$MgCl_2$
Major histocomaptibiliy class II	MHCII
Malondialdehyde	MDA
Minimal erythemal dose	MED
Messenger ribonucleic acid	mRNA
Molony murine leukaemia virus reverse transcriptase	MMLV
Mouse double minute-2	MDM-2
Nicotinamide adenine dinucleotide phosphate	NADPH
Non melanoma skin cancer	NMSC
Nuclear factor kappa B	NFκB
Nucleotide excision repair	NER
Penicillin-streptomycin	Pen-strep
Phenylmethylsulfonyl fluoride	PMSF
Phorbal-12-myristate-13-acetate	PMA
Phosphate buffered saline	PBS
Phospholipid glutathione peroxidase	PHGPX
Polymerase chain reaction	PCR
Polyvinyldifluoride	PDVF
Protein kinase C	PKC
Reactive oxygen species	ROS
Ribonucleic acid	RNA
SDS-polyacrylamide gel electrophoresis	SDS-PAGE
Selenocysteine insertion element	SECIS
Sodium dodecyl sulphate	SDS

Squamous cell carcinoma	SCC
Selenomethionine	SM
Sodium selenite	SS
Standard deviation	SD
Standard error of the mean	SEM
Superoxide dismutase	SOD
T4 polynucleotide kinase	PNK
Tetrasodium ethylenediaminetetracetic acid	EDTA
Thiobarbituric acid	TBA
Thiobarbituric acid reactive substances	TBARS
Thioredoxin reductase	TR
Transfer RNA	tRNA
Transforming growth factor-β	TGF-β
Tris-buffered saline: Tween 20	TBS:T
Tumour necrosis factor-α	TNF-α
Ultraviolet radiation	UV
Volume/volume	v/v
Weight/volume	w/v

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Chapter 1

Introduction

1.1 The skin.

The skin is the largest organ of the body. It is in continual contact with the external environment and therefore, in addition to controlling water loss and temperature regulation, it encounters a wide range of potentially damaging factors such as ultraviolet (UV) radiation, chemicals and micro-organisms. The skin was originally considered to function purely as a physical barrier; however it is now known to be a highly immunoreactive organ, which defends the body from invading pathogens. The skin is also capable of self repair. The skin as an immunoreactive entity complete with its associated lymphoid tissues, was first described nearly 20 years ago (Streilein, 1978).

The skin is divided into two main compartments they are: the epidermis and dermis, and are separated from each other by the basement membrane (Fig 1.11).

1.1.1 Keratinocytes.

Keratinocytes constitute 92% of the cells in the epidermis forming a stratified multi-layered epithelium. Keratinocytes provide the physical barrier properties of the epidermis and accomplish its repair and regeneration. The keratinocytes situated adjacent to the basement membrane are predominately undifferentiated, rapidly proliferating stem cells. As keratinocytes become committed to differentiate, they detach from the basement membrane, lose their capacity to proliferate and migrate from the basal layers towards the stratum corneum. Terminally differentiated keratinocytes are finally shed from the stratum corneum as dead cornified squames (reviewed in Watt, 1989). In addition to forming a physical barrier, keratinocytes have an important immunoregulatory role and keratinocytes in culture can be stimulated to secrete many cytokines. The functions of some of these cytokines will be discussed later in the chapter.

1.1.2 Langerhans' cells and other immune cell types of the skin.

In addition to keratinocytes, the other components of the skin include Langerhans' cells (LCs), leukocytes, endothelial cells, melanocytes and mast cells. Langerhans' cells are cluster of differentiation (CD) la+, major histocompatibility (MHC) Class II dendritic cells derived from the bone marrow. They form a semi-continuous network between epidermal keratinocytes and are the main antigen presenting cell (APC) of the skin. Langerhans' cells are defined by their dendritic morphology and possession of a unique intracytoplasmic organelle called a Birbeck granule, the function of which remains unclear (Birbeck *et al*, 1961). Unlike keratinocytes, LCs are a migratory population of cells and do not permanently reside in the epidermis. Immature LCs in the epidermis develop the capacity to become potent APCs, after migration from the skin to the draining lymph nodes where they process and present antigens to naive T cells (Streilein *et al*, 1990). In a secondary immune response, antigen presentation to memory T cells may then occur within the skin by LCs or macrophages (Streilein *et al*, 1990). Invading pathogens, contact sensitisers or exposure to ultraviolet light can all induce LCs to migrate from the epidermis.

T cells, macrophages and other leukocytes enter the dermal and epidermal compartments from dermal blood vessels. The majority of T cells within the dermis comprise equal numbers of T helper cell (CD4+) and cytotoxic T cell (CD8+) subsets and are located in the perivascular region. In contrast, only 2% of the T cells in normal skin are found in the epidermis and they are predominately CD8+ (Bos *et al*, 1987).

1.1.3 Melanocytes.

Melanocytes are highly differentiated neural crest-derived cells which are present in the basal layer of the epidermis. Melanocytes are responsible for basal skin pigmentation and for tanning. However their primary function is to protect the skin from UV irradiation, they do this by the production and transfer of melanin to keratinocytes (reviewed by Gilchrest and Eller, 1999). Melanin consists of two types, eumelanin and phaeomelanin. Melanin is produced in specialised intracellular organelles called melanosomes, following production; the melanin is transported through the dendritic

processes of the melanocytes to the surrounding keratinocytes. Following exposure to UV radiation, the number of melanosomes and their production of melanin greatly increases (Yaar and Gilchrest, 1991). Melanin can directly absorb UV photons thereby dissipating the otherwise injurious energy as heat. Melanin can also absorb free radical species generated by the interaction of UV photons with cellular lipids. When stimulated, melanocytes respond to many cytokines and can also secrete several cytokines including; Interleukin (IL)-1α and IL–β, transforming growth factor-β (TGF-β), IL-8 and monocyte chemotactic protein-1 (reviewed by Thody, 1995). In addition, intracellular adhesion molecule-1 (ICAM-1) can be induced on the surface of melanocytes, thus promoting cell-cell interactions.

1.2 Sunlight and skin cancer.

1.2.1 Historical note.

Solar radiation has been worshipped since ancient times for its health inducing properties. The Egyptians believed that the sun's energy was from *Aton Ra*, the sun god dating from the Fifth Dynasty 2750 BC. Helios the Greek god of sun and light has given his name to heliotherapy, which means healing by exposure to natural sunlight. Herodotus, in 525 BC, observed that the strength of the skull was related to sunlight exposure. Sunbathing was recommended at this time for a range of conditions such as epilepsy, jaundice and obesity. During the eighteen and nineteenth centuries heliotherapy was used in the treatment of rickets (Diffey, 1984). Sunlight is essential for the photochemical formation of vitamin D3 in the skin, which is necessary for bone formation. It is still used for the treatment of certain skin diseases such as psoriasis.

The Danish physician Niels Finsen (1860-1904) realised the importance of the UV component of sunlight, with respect to skin disease in the late nineteenth century. In his 1893 publication *'On the influence of light and the skin'* and in later published articles Finsen stressed that it was the UV radiation not heat, from the solar spectrum that was responsible for sunburn. Finsen went on to win the Nobel Prize for Medicine for his work on the treatment of lupus vulgaris with UV radiation.

The advent of cheap air travel and increased leisure time has meant that more people can now travel to sunny climates on a regular basis. There has been a gradual increase in the availability and popularity of artificial tanning devices. It is thought that this 'suntan seeking' behaviour is largely responsible for the rapidly rising incidence of skin cancer in Europe and North America.

1.2.2 The electromagnetic spectrum.

The sun is the major source of human exposure to UV radiation. Approximately 50% of the sun's energy that reaches the earth's surface, comes from the visible portion of the electromagnetic spectrum and only 5% is from the UV component of the electromagnetic spectrum. The UV part of the spectrum is subdivided into UVC (200-290 nm), UVB (290-315 nm) and UVA (315-400 nm) (Fig 1.1). UVC radiation is absorbed completely by the stratospheric ozone layers and is therefore not of biological relevance (Diffey, 1984). UVB radiation is only partly absorbed by the ozone layer and UVA radiation is not absorbed at all. The UV spectrum of the sun at ground level is dependent on geographical location, the time of day, month of year and also on the weather conditions, level of air pollution and reflection from the Earth's surface. An example of the solar spectrum in the UK on a clear summer day, shows that UVB and UVA can be measured only (Fig 1.2).

1.2.3 Environmental considerations.

There is concern regarding the depletion of the stratospheric ozone and the concomitant increase in levels of genotoxic UVB radiation, which is predicted to increase the risk of skin cancer in the future (Slaper *et al*, 1996). Ozone is the primary absorber of UV light in the atmosphere. It blocks all UVC from reaching the Earth's surface, 90% of UVB, but very little UVA. Inert gas propellants from aerosol cans (chlorofluorocarbons) and high-flying jets have been blamed for the depletion of the ozone layer. Since 1985 seasonal fluctuations in the ozone layer have been observed regularly in Antarctica.

Figure 1.1: The electromagnetic spectrum.

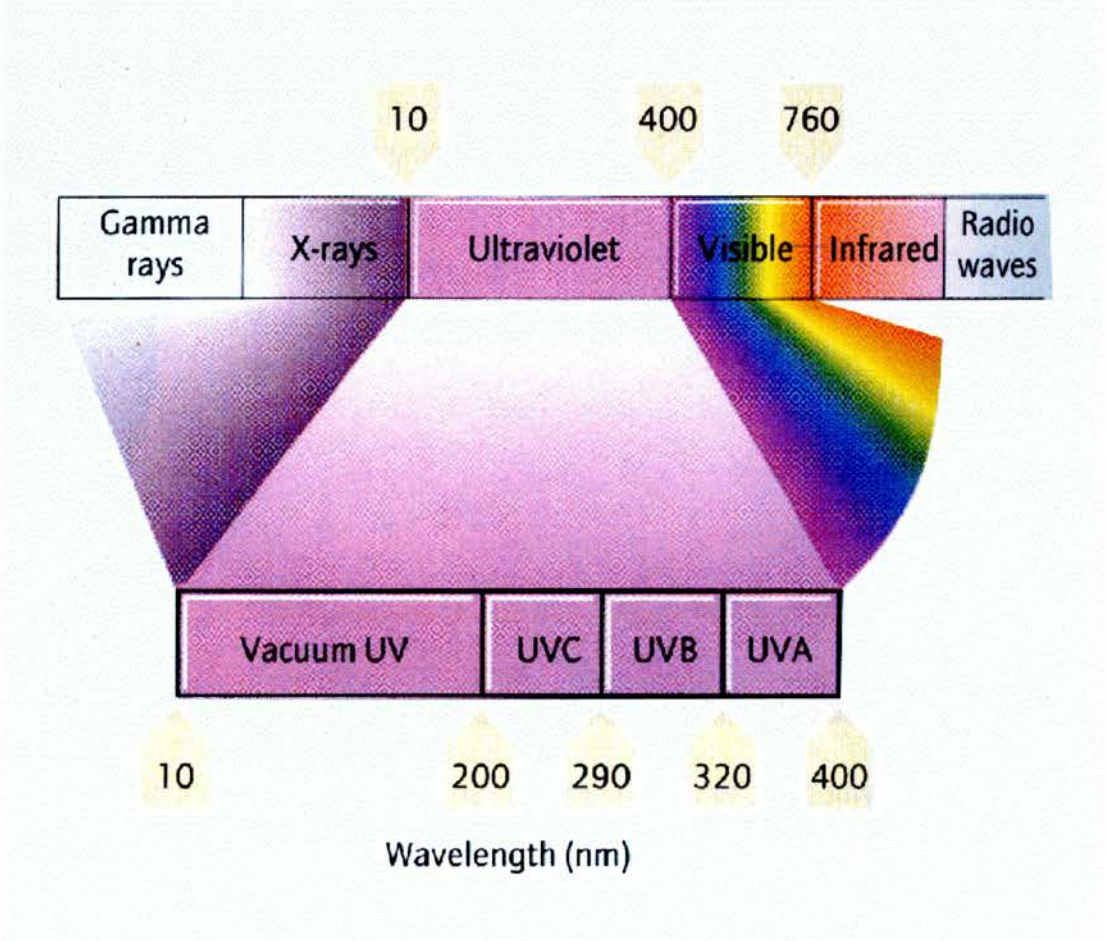
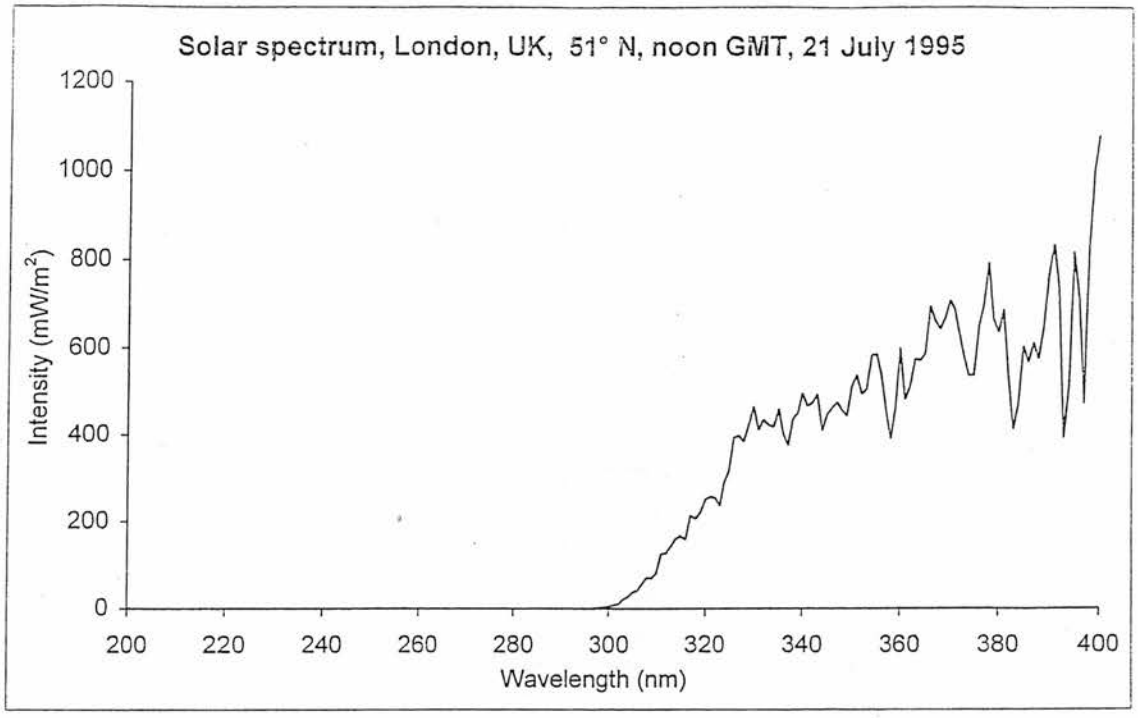


Figure 1.2: Typical solar spectral output in the UK on a clear, summer day.



and in some parts of the Northern hemisphere where there is minimal air pollution (Kerr, 1992; Hilsenrath *et al*, 1992). It is believed that any reduction in the atmospheric ozone layer will lead to increased environmental exposure, in particular to UVB which reaches the Earth's surface in amounts inversely proportional to the concentration of atmospheric ozone (Longstreth *et al*, 1998). International commitment has resulted in a decrease in the production and emission of ozone-damaging chemicals, which may eventually lead to a recovery of the ozone layer.

1.2.4 Chromophores present in the skin.

The longer the wavelength of light, the deeper into the skin it can penetrate. Therefore, UVA can penetrates more deeply into the dermis than UVB. Chromophores in the skin absorb the UV radiation and are altered or damaged by the energy they absorb. The major chromophores present in the

skin are DNA, proteins and urocanic acid. The effect of UV on deoxyribonucleic acid (DNA) is discussed in detail later in the chapter. Absorption of photons by proteins can lead to the formation of protein-protein cross-links and protein-DNA cross-links.

Urocanic acid is produced by the de-amination of the essential amino acid histidine. The skin does not contain the enzyme urocanase to catabolize urocanic acid, therefore the skin contains a high level of the latter. In the epidermis, urocanic acid exists as a trans-form. Exposure to UV radiation isomerises urocanic acid from the trans-form to the cis-form, which is thought to be an important mediator of UV-induced regulation of cellular responses and immunosuppression in the skin (reviewed in Beissert and Granstein, 1995; Norval *et al*, 1995; Kondo *et al*, 1995).

When the skin is exposed to UV radiation there is an increase in the release of arachidonic acid from membrane bound phospholipids, through the activation of phospholipase A_2 and phospholipase C. The subsequent increase in prostaglandin, is thought to be involved in the erythema reaction following exposure to UV.

1.3 Epidemiology of skin cancer.

It is widely accepted that UV radiation is carcinogenic in humans and can produce photochemical changes in superficial tissues, resulting in acute and chronic adverse health effects. In 1894 the dermatologist Unna Firt recognised the relationship between skin cancer and exposure to sunlight (reviewed in Diffey, 1984). Acute effects of exposure to sunlight include; erythema resulting from vasodilatation of the blood vessels in the skin, sunburn, blistering, photokeratitis and conjunctivitis. Chronic effects include; premature photoaging, lens cataracts, malignant and benign tumours, and immunosuppression.

UVB has long been recognised as carcinogenic in experimental animals, and there is increasing evidence that UVA, which penetrates more deeply into the skin also contributes to photocarcinogenesis. The wavelength dependence (action spectrum) of UV-induced skin cancers has been estimated (de Gruijl, 1993). These studies on wavelength dependence,

demonstrate that the UVA contribution to the carcinogenicity of sunlight is approximately 20% with UVB causing 80%.

The majority of experimental models of UV-induced carcinogenesis have been carried out using hairless mice, which mainly develop squamous cell carcinomas (SCC) (de Gruijl and Forbes, 1995). The action spectrum for the development of SCCs in the hairless mouse, as well as that for non-melanoma skin cancer development in human skin, closely resemble the human erythemal action spectrum (Parrish *et al*, 1982). The action spectra show that UVB radiation is the most efficient wavelength at inducing skin tumours, however a deleterious role for UVA in skin carcinogenesis has also been demonstrated. This is an important factor to consider, due to the high intensity of UVA in sunlight and the inability of some sunscreens to block UVA wavelength effectively.

The incidence of the three main types of cutaneous malignancies; basal cell carcinoma (BCC), SCC, and malignant melanoma, has nearly doubled between 1980-1990 (Devessa *et al*, 1995). The number of skin cancer-related deaths has risen by around 50% over the last 15 years.

Non-melanoma skin cancers (NMSC) are the most common type of skin tumours to occur, they are generally curable, and death is uncommon. The occurrence of NMSC is related to chronic UV exposure and it is therefore commoner in fair skinned individuals (Stern and Momtaz, 1984), albinos and individuals with DNA repair defect disease *xeroderma pigmentosum*. Some of the apparent increased incidence of skin cancers are thought to be due to better reporting of cases.

1.3.1 Basal cell carcinoma.

BCCs are slow-growing, locally invasive malignant epidermal skin tumours. BCCs, represent the most common type of skin cancer. It is estimated that 40,000 are currently annually diagnosed in the UK. The most significant etiologic factor is chronic exposure to UV radiation, consequently the head and neck are the most commonly involved sites (Cheronsky, 1978). The clinical appearance and morphology of BCCs are diverse ranging from; nodular to cystic, ulcerated, superficial, keratotic and pigmented. They are

rarely fatal and metastasis is rare (Lo *et al*, 1991). However, BCCs can result in significant morbidity from local tissue destruction, particularly on the head and neck if not detected early. A 5% increase in incidence of these tumours per annum has been detected. The incidence of BCC rises with increasing age. With an ageing population it is estimated that the incidence of BCC in the UK will rise further over the next decade. This could have major economic implications.

1.3.2 Squamous cell carcinoma.

SCCs are malignant tumours arising from keratinocytes in the epidermis or from mucous membranes. SCC's are locally invasive and have the potential to metastasise to other organs of the body. SCC's represent the second most common skin cancer. Approximately, 10,000 are diagnosed annually in the UK at present. In many countries as well as the UK the incidence is rising. SCC's are more common and more aggressive in patients who are immunosuppressed, such as those receiving immunosuppressive medication following renal or other organ transplants. SCC's usually present as an indurated nodular warty, keratinising tumour which can become ulcerated, typical sites being the ears, head and neck.

1.3.3 Malignant melanoma.

Malignant melanomas are malignant tumours derived from melanocytes, they are the leading cause of death from skin disorders. Melanomas typically arise from pre-existing melanocyte naevi. They are usually, but not always, dark brown/black, with irregular pigment and borders. Patients usually notice change in shape, size or colour of pre-existing naevi. The mortality rates from melanoma have been rising over the last 50 years, (Weinstock, 1993) with a rapidly rising incidence of 7% per annum in the UK in the past decade. 5,000 cases are diagnosed annually in the UK. Unfortunately the past decade has also seen an increase mortality from malignant melanomas, of 2% per annum in England and Wales. It is thought that the risk of melanoma may be associated with short periods of intense exposure to UV radiation and possibly acute episodes of sunburn especially in childhood (Elwood, 1992a and b).

1.4 Adverse effects of UV radiation exposure on the skin.

1.4.1 Induction of cell death.

Exposure to UV can induce cell death in the skin. There are two distinct forms of cell death, termed necrosis and apoptosis. Necrosis is a passive form of cell death resulting from acute cellular injury, in which cells tend to swell and then lyse. Apoptosis in comparison is an active form of cell death, with specific morphological features which differ from necrosis (Kerr *et al*, 1972). The nucleus and cytoplasm shrink (condensation of the chromatin occurs), the nucleus becomes pyknotic and the cell fragments into membrane -enclosed fragments called apoptotic bodies (Kerr *et al*, 1994). Within tissues the apoptotic bodies are rapidly recognised and phagocytosed by neighbouring cells. Then the apoptotic bodies are degraded within the cells lysosomes, resulting in their effective removal. As the cells are removed quickly and do not lyse, no cellular contents leak and there is no inflammatory response, unlike necrotic cell death. Apoptosis is often labelled programmed cell death, as it occurs during development and differentiation of tissues and mediates the changes in organ structure and function (Sen, 1992). It is also the major mechanism by which homeostasis of a number of physiological systems in the body can be regulated. In the skin it is thought that apoptosis is a ubiquitous process, that is important in regulating epidermal growth (Weedon, 1990). Apoptosis usually effects individual cells and not whole populations, unlike necrosis.

There are many factors which induce apoptosis in different cell types they include; tumour necrosis factor (TNF)-α, tumour growth factor (TGF)-β, retinoids, calcium, growth factor withdrawal, heat, cold, UV radiation and oxidative stress. Other factors can inhibit apoptosis including; growth factors, interleukin (IL)-2, IL-3, IL-4, IL-6, CD40 ligand, zinc, antioxidants and calpain inhibitors. Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) can induce cells to undergo apoptosis (Lennon *et al*, 1991). The intensity of stimuli is thought to be important in the induction of apoptosis, above a certain threshold of stimulus and cells undergo cell death by necrosis. This has been demonstrated with H_2O_2 (Lennon *et al*, 1991).

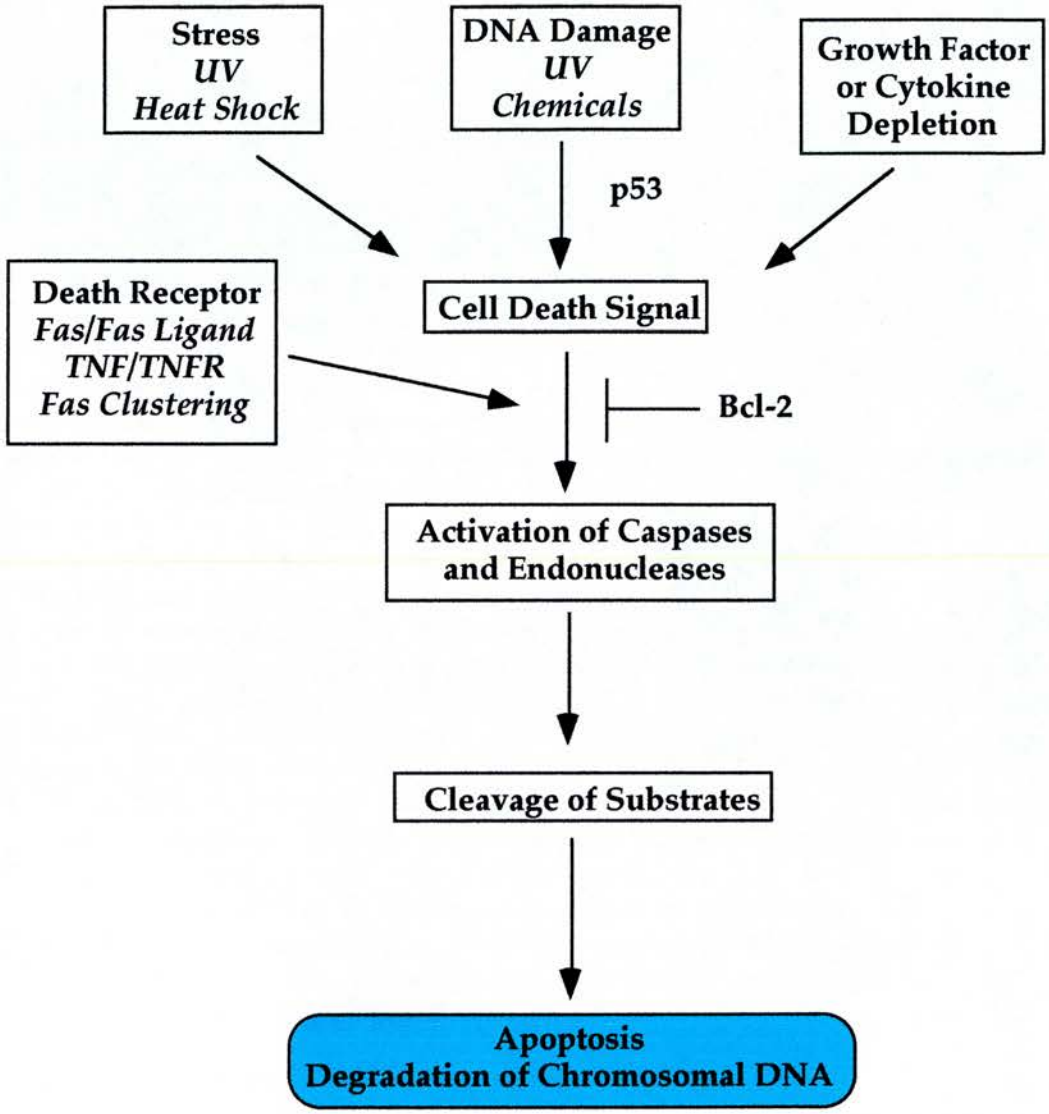
Biochemical changes specific to apoptotic cells also occur, these involve the activation of catabolic enzymes. A class of highly specific proteases also appear to have an important role in apoptosis. Proteases of this class are related to mammalian interleukin-1β converting enzyme (ICE) and to cell death gene (CED)-3. All of these proteins possess the amino acid cysteine in their active site and cleave their target proteins at specific aspartic acids and are called caspases. The caspase proteins can activate other members of the family, so that a network of caspase activation occurs. The caspases have different activation signals for example, caspase-8 activates caspase-1 which in turn activates caspase-3 during Fas-induced apoptosis (Fig 1.3).

There are two main pathways of apoptosis. One of these is the Fas pathway; Fas is a transmembrane receptor and a member of the TNF receptor superfamily. FasL the ligand for Fas, is a transmembrane protein that belongs to the TNF family. When FasL binds to Fas at the cell surface, Fas trimerises and then Fas-associated protein with death domain (FADD) associates with the receptor trimer via a homotypic death domain interaction (Takahashi *et al*, 1994). Other death domain containing-proteins which interact with the Fas receptor are MORT1 and RIP. Other ICE-like proteases are then recruited i.e. FLICE or MACH. Exposure to UVB can also lead to Fas trimerisation without FasL binding (Aragane *et al*, 1998) (Fig 1.4).

Another apoptosis pathway related to the one mediated by Fas involves the TNF receptor, it associates with TRADD (TNF receptor 1-associated death domain), which then recruits FADD (Greenblatt and Elias, 1992). Then FADD concomitantly recruits caspase-8 in both pathways; once activated it can cleave a number of nuclear protein substrates, resulting in DNA fragmentation and the activation of other caspases.

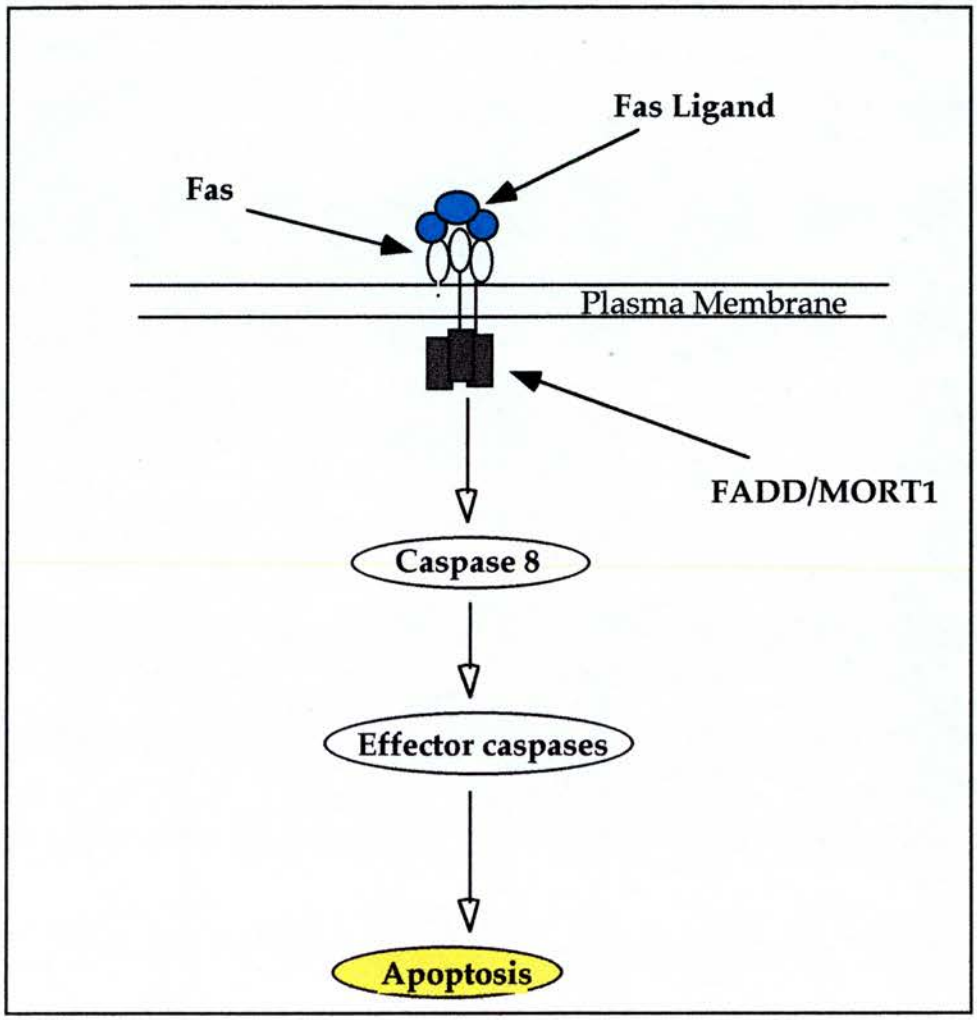
The final pathway for induction of apoptosis is via p53. The human p53 gene encodes a nuclear phosphoprotein, with a molecular mass of 53,000. It is one of the most frequently mutated genes known, with more than 50% of human malignancies containing altered p53 genes (Hollstein *et al*, 1991).

Figure 1.3: Overall pathway of apoptosis.



The cell receives the apoptotic-inducing signal and either the Fas, TNF or p53 pathway leading to apoptosis is activated. This leads to the activation of caspases within the cell and cleavage of cellular DNA and proteins until the cell is divided into apoptotic bodies which are phagocytosed by other cells (adapted from Teraki and Shiohara, 1999).

Figure 1.4: Fas-mediated apoptosis.

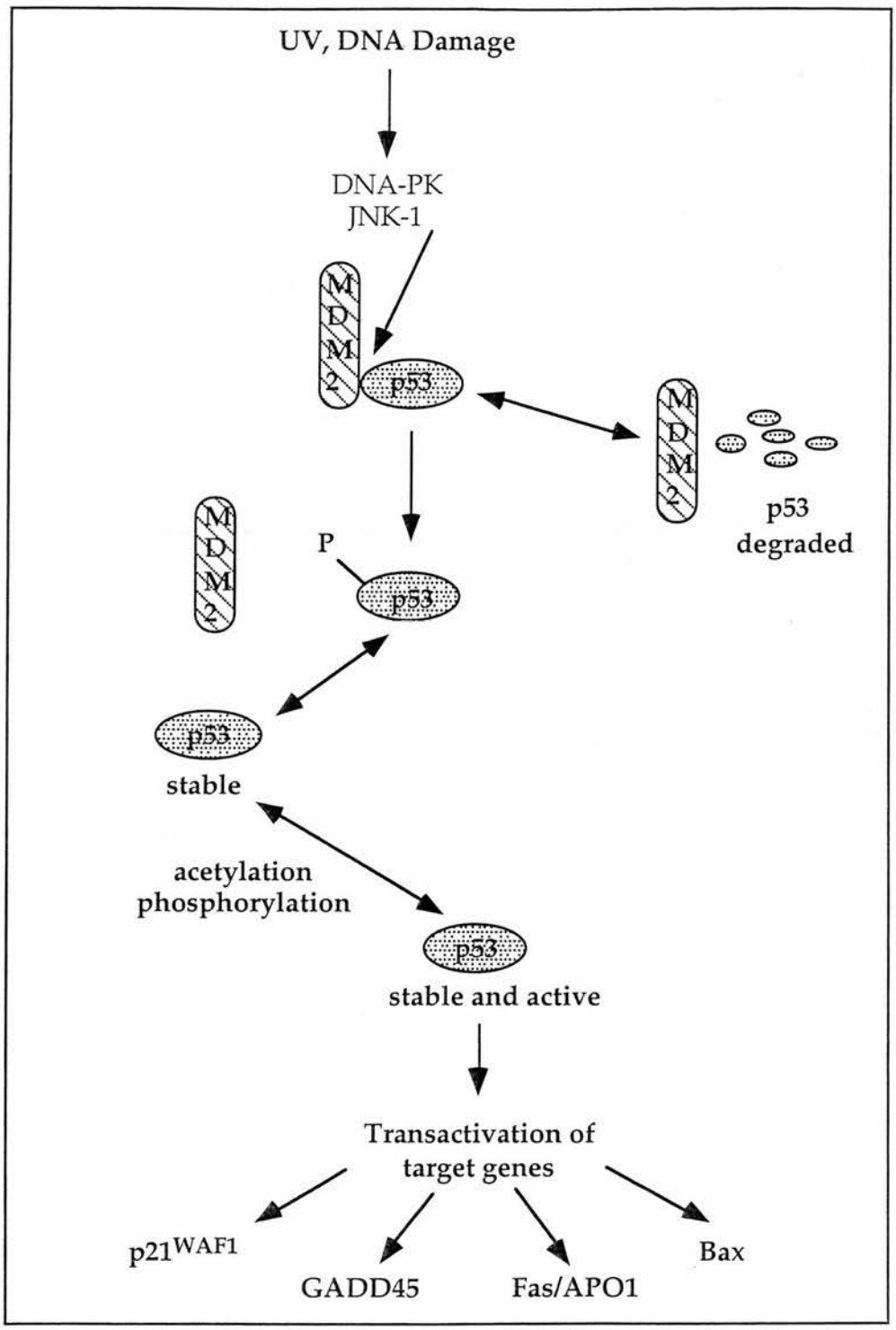


Either FasL binds to Fas or UV radiation activates Fas thus leading to the trimerisation of Fas. This leads to the recruitment of FADD or MORT1 which in turn leads to the activation of caspases and fragmentation of the cell into apoptotic bodies (adapted from Teraki and Shiohara, 1999).

The p53 tumour suppressor gene is mutated in the germline of individuals with Li-Fraumeni syndrome. These individuals have a high risk of developing cancerous growths, greater than 50% Li-Fraumeni patients will develop tumours by age 30 (Malkin *et al*, 1990). The p53 protein functions as a tumour suppressor by inducing growth arrest, senescence, or apoptosis in response to a variety of cellular stresses. These cellular stresses include; exposure to DNA damaging agents, hypoxia, nucleotide depletion, and oncogene activation. Apoptotic responses protect the cell from uncontrolled proliferation and neoplastic transformation. The N-terminal transactivation domain of p53 is important for the stability of the protein in the cell. The regulatory protein mouse double minute 2 (MDM2) can bind to this region and block p53 transactivation, by preventing phosphorylation of this region (Momand *et al*, 1992), as a result p53 is degraded (Midgley and Lane, 1997). The basic C-terminal region of p53 is able to bind single stranded DNA. The half life of wild type p53 in unstressed cells is very short 20-30 minutes only. Within stressed cells the p53 protein is stabilised and it's DNA binding activity increases, allowing it to mediate cell cycle arrest or apoptosis. The protein MDM2 is thought to destabilise p53 and when it dissociates from the p53 protein, p53 has a longer half life (Fig 1.5). After DNA damage p53 becomes phosphorylated and MDM2 binding is decreased (Shieh *et al*, 1997, reviewed in Burns and El-Deiry, 1999). Phosphorylation at serine 389 and acetylation of the C-terminal region of p53 increases it's DNA binding activity, this is thought to occur due to allostearic alteration of the p53 protein allowing it to bind DNA (Hupp and Lane, 1994) (Fig 1.6). Damage to DNA induced by exposure of cells to UV has been demonstrated to lead to phosphorylation of serine 389 (Lu *et al*, 1998). The UV-induced protein kinase JNK-1 phosphorylates p53 at serine 34, this is within the region where MDM2 binds (Milne *et al*, 1995). The levels of protein for p53 do not correlate with p53 activity, the p53 protein appears to have to be activated.

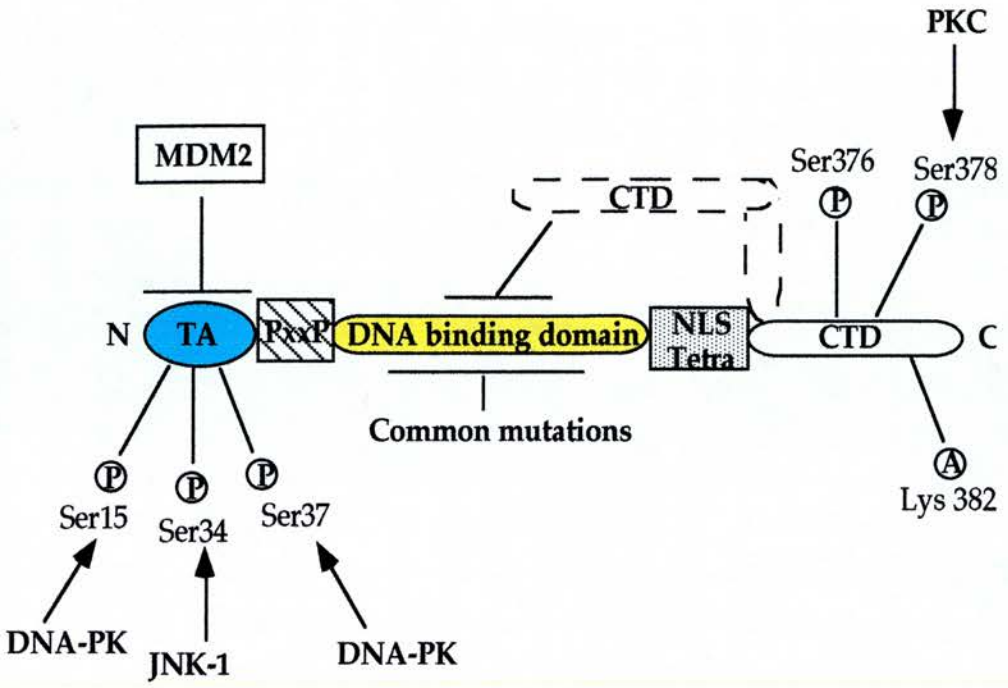
Other kinases can also phosphorylate p53 at various serine residues, these include; at the C-terminus, protein kinase C (PKC) which phosphorylates serine 378 and cyclin dependent kinase 2 (CDK2) which modifies the tetramerisation domain. DNA protein kinase (DNA-PK) which phosphorylates serine 37 and 15 in the N terminal binding domain. Therefore activation of specific kinases can stimulate p53 towards different functions (reviewed in Burns and El-Deiry, 1999).

Figure 1.5: Pathway of p53 activation.



p53 binds to MDM2 and is constantly degraded, if p53 is phosphorylated this prevents MDM2 binding and so stabilises the protein. The stabilised p53 is then phosphorylated or acetylated again which results in a conformation change and allows the protein to bind DNA and activate downstream genes (adapted from Burns and El-Deiry, 1999).

Figure 1.6: Structure of p53 protein.



MDM2 binds p53 at the N-terminal transactivation domain (TA), several phosphorylation sites are present which can be phosphorylated to prevent MDM2 binding. The protein also contains a proline rich region (PxxP) and a tetramerization domain (TETRA). Phosphorylation of the C-terminal domain (CTD) can alter the shape of the protein to uncover the central DNA binding domain. Most of the mutations found within tumour cells occur in the DNA binding domain (adapted from Burns and El-Deiry, 1999).

If the intensity of UV radiation is low then there is limited DNA damage and p53 activates growth arrest genes, the cell then undergoes cell cycle arrest at G1, the damaged DNA is repaired and the cell is allowed to progress through the cell cycle (Lane, 1992; Cox and Lane, 1995). If the intensity of irradiation is high then a substantial level of DNA damage results and p53 signals the cell to undergo apoptosis, which will prevent the mutated DNA from being passed on to subsequent generations of daughter cells. Cells which are actively replicating during the exposure to UV are more likely to undergo apoptosis, this appears to be due to p53 DNA proof-reading capability (Danno and Horio, 1987). Once activated p53 can bind directly to

single stranded DNA and interact with the DNA replication machinery (Cox *et al*, 1995). In this situation p53 can then regulate the transcription of genes involved in cell cycle arrest (Lui and Pelling, 1995) and apoptosis (Selvakumaran *et al*, 1994). Examples of the genes which p53 can control the transcription of are $p21^{WAF1/CIP1}$ (EL-Deiry *et al*, 1994), GADD45 (Zhan et al, 1994) and MDM2 (EL-Deiry *et al*, 1994) these genes appear to be involved in the p53 response to DNA damage. The protein $p21^{WAF1}$ is a potent inhibitor of cyclin dependent kinase activity (Xiong *et al*, 1993), it can also interfere directly with DNA synthesis (Waga *et al*, 1994). The protein GADD45 can also suppress the cell cycle and stimulate nucleotide excision repair (Smith *et al*, 1995, Li *et al*, 1997). It is known that p53 can down regulate Bcl-2 (B-cell leukaemia-2) expression and increases Bax expression, these actions could increase the rate of apoptosis (Miyashita *et al*, 1994, reviewed in Hoffman and Liebermann, 1994).

Many proteins of the Bcl-2 family can prevent apoptosis e.g. Bcl-2, Bcl-Xl, Bcl-w. Other members of the Bcl family can drive apoptosis such as Bax, Bik, Bak, Bad, Bid. All of these proteins commonly co-exist within cells and it is thought that the ratio of these proteins (e.g. Bcl-2 to Bax) regulates the cells susceptibility to undergo apoptosis (Chao and Korsmeyer, 1998). Bcl-2 has been demonstrated to respond to the redox potential of cells and may have antioxidant actions (reviewed in Kroemer, 1997).

The importance of UVB as an initiator of tumourogenesis, is thought to result from its ability to cause mutations in genes which control the cell cycle such as; proto-oncogenes for example Ras and tumour suppressor genes such as p53. Cells in the epidermis accumulate mutations over years of exposure to sunlight and the resulting dysfunctional genes can eventually lead to a malignant transformation. The majority of the mutations in p53 which are found in human tumours are located in the DNA binding region of the protein. This region contains ten cysteine residues, eight of which are conserved, implying that they are important for the function of p53. The cysteine residues form a loop-sheet-helix motif around a zinc atom, which forms the DNA-binding surface of the p53. Mutations can either replace or alter the cysteine residues, which will disrupt the correct conformation of p53, thus abolishing wild type p53 activity. Over 90% of SCC's in the USA were found to have mutations within the p53 gene (Brash *et al*, 1991). In

BCC's mutations in p53 are also found but only in 50% of tumours (Rady *et al*, 1992; Zeigler *et al*, 1993). BCCs have been demonstrated to over express p53 protein, it is thought that the p53 is mutated and has an extended half life. The mutation is thought to prevent cells from halting in cycle arrest and proceeding through apoptosis, as $p21^{waf/cip1}$ is expressed in normal epidermis following exposure to UVB, but not in neoplastic epidermis (Urano *et al*, 1995; Inohara *et al*, 1996). The absence of $p21^{waf/cip1}$ will allow tumour cells to grow in a unregulated manner and also escape the apoptotic pathway (reviewed in Brash *et al*, 1996). Therefore sunlight can promote skin cancer by first of all initiating a mutation in the DNA coding for p53, then by promoting the growth of the altered cells by inactivating the p53 protein. As a result altered cells do not undergo apoptosis on exposure to sunlight and do not halt in growth arrest to repair their DNA.

Terminal differentiation of keratinocytes is thought to be a specialised form of apoptosis (Maruoka *et al*, 1997), because there are similarities between terminally differentiating keratinocytes and apoptotic cells; for example, granular keratinocytes show signs of endonuclease activation and DNA fragmentation (McCall and Cohen, 1991). Recently however it has been suggested that terminal differentiation, whilst similar to apoptosis is a distinct mechanism. The differences include: the extended time needed for keratinocytes to differentiate (days instead of hours), differentiating keratinocytes do not fragment, unlike apoptotic cells and finally differentiation specific keratin genes are turned on, which are not expressed within apoptotic cells (Gandarillas *et al*, 1999) Apoptotic keratinocytes are characterised by a condensed and basophilic nucleus and eosinophilic homogenisation of the cytoplasm, they are often referred to being dyskeratotic cells or sunburn cells (Young, 1987). Apoptotic cells in culture also loose their cell-cell contacts, round up and develop cell surface blebs before detaching from the cell monolayer (Malorni *et al*, 1994). Surface blebbing and rounding up can be decreased by the addition of antioxidants such as α-tocopherol (Malorni *et al*, 1996) and glutathione (Godar, 1999). Keratinocytes can express Fas, FasL, TNF receptor, TNF and p53 so can undergo apoptosis through all these described pathways.

1.4.2 Ultraviolet Radiation induced DNA damage.

As discussed earlier in this chapter the effects of UV radiation are based on the absorption of the radiant energy by an appropriate molecule (chromophore). The many of the biological effects of UV exposure are thought to be causally related the absorption of UV by DNA. The role of UV radiation in initiation of skin carcinogenesis is well established and results from photochemical modification of DNA. The wavelengths of UV that are carcinogenic for human cells correspond to the absorption spectrum of DNA (Sutherland *et al*, 1980).

DNA is constantly damaged by a variety of chemical and physical agents such as UV radiation. All cells possess mechanisms for repairing DNA damage. Most UV radiation-induced DNA damage is efficiently repaired, but if the damage burden is too great these DNA repair mechanisms become overwhelmed and some damage becomes fixed in the form of permanent mutations.

Absorption of UV photons by a DNA molecule produces an excited state, that is followed by a rearrangement of electrons to form a variety of photoproducts. DNA absorbs UV radiation in the range 230-300nm. The main mechanisms of UV radiation-induced DNA damage are wavelength-dependent. Ultraviolet radiation may damage DNA directly as detected with shorter wavelengths of UVC or UVB, or indirectly; longer wavelength UVA acting through the formation of reactive oxygen species (ROS). The most superficial keratinocytes of the epidermis, which receive the greatest UV exposure, are committed to terminal differentiation as they move into the stratum corneum. Therefore injury to these cells is not highly significant. However, mutation of the basal-layer stem cells may result in the development of malignancy. The process of excision repair is essential for the repair of these lesions.

Direct DNA damage.

Cyclobutane pyrimidine dimers (CPDs) are the predominant UV photoproduct, accounting for approximately 85% of DNA damage induced by UVC and UVB.

The formation of CPD involves redistribution of electrons in adjacent pyrimidines and single-bond ring closures at the 5- and 6- positions, giving rise to covalent cyclobutane linkage (Fig 1.7). CPDs are bulky DNA lesions involving the chemical bonding of adjacent thymine or cytosine base pairs to form cyclobutane rings using energy from UV radiation. Approximately 50% are found at TT sequences, 40% at TC/CT, and 10% at CC sequences.

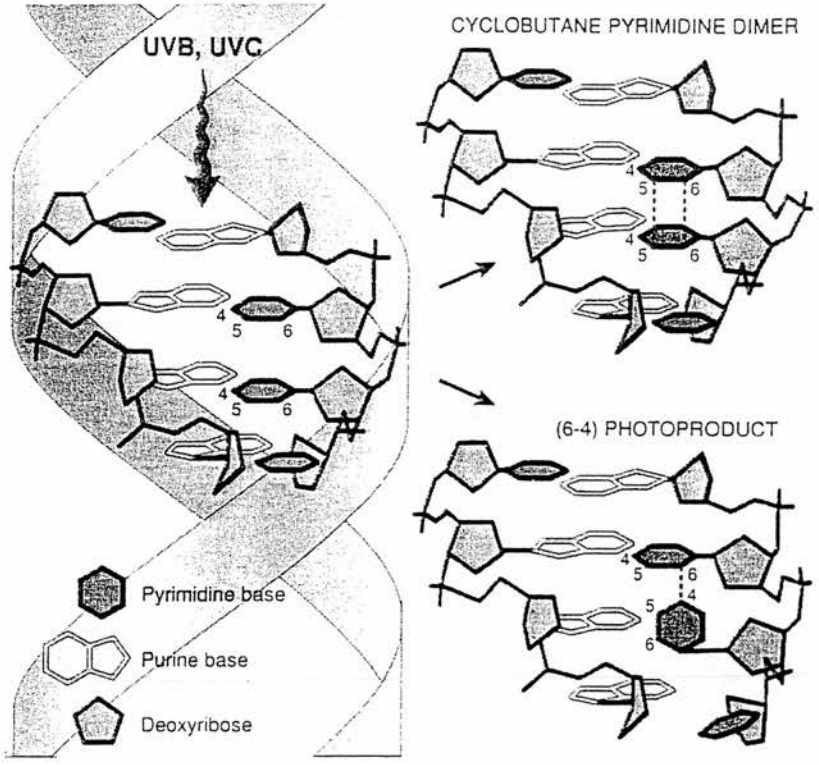
The 6-4 pyrimidine-pyrimidone photoproduct is the second most common UVB photoproduct, accounting for between 10-30% of UV induced lesions. It is formed by reaction of the carbon-carbon double bond at the 5-position of one pyrimidine with the carbon-nitrogen double bond of an adjacent pyrimidine, resulting in covalent linkage between the 6- and 4- positions of the respective pyrimidines (Fig 1.7).

Dewar isomers are formed through the photoisomerization of 6-4 photoproducts. This is stimulated most efficiently by UV wavelengths of around 320 nm. Consequently it has been suggested that the majority of 6-4 photoproducts will be converted to Dewar isomers upon exposure to solar UV radiation (Clingen *et al*, 1995).

Direct types of DNA damage are found in the DNA from human BCCs and SCCs. Also patients with the hereditary condition *xeroderma pigmentosum* are 2000 times more likely than normal individuals to develop skin carcinomas (Lambert *et al*, 1995). These patients are classically defective in the repair of direct DNA damage (Cleaver, 1968), providing additional evidence of the strong link between this type of DNA damage and skin cancer.

In contrast to CPDs and 6-4 photoproducts there is relatively little information regarding the biological effects of Dewar isomers (Mitchell and Nairn, 1988). The mutagenicity of Dewar isomers has not yet been established in mammalian cells. However, transfecting single-strand DNA vectors containing a single site-specific photoproduct into *Escherichia coli* or *Saccharomyces Cerevisae* has shown the TC Dewar isomer to be more mutagenic that the TC 6-4 photoproduct.

Figure 1.7: Two major direct photoproducts formed after UV exposure.



The CPD (top right) is formed when the energy from UV opens the double bonds between Carbon (C) atoms 5 and 6 of two neighbouring pyrimidine rings and forms new bonds between them. The second major photoproduct is the 6-4 photoproduct (bottom right). Again the double bonds of two adjacent pyrimidines are broken however when the new bonds are being formed one pyrimidine rotates and the new bonds form between position C4 on one pyrimidine and position C6 of the other (Adapted from Wikonal and Brash, 1999).

Indirect DNA damage.

Indirect forms of DNA damage induced by UV radiation include: oxidised or hydrated pyrimidines (cytosine photohydrates and thymine glycols), oxidised purines (8-hydroxyguanosine), single-strand breaks and DNA protein cross-links (Jen *et al*, 1997).

Once a non-DNA chromophore is excited by the absorption of a photon, it must transfer its energy to DNA in order to produce chemical modification of DNA. This process may involve direct energy transfer between the sensitiser and one of the DNA bases, which results from absorption of short wavelength UVB and UVC or via the production of chemically reactive intermediates such as reactive oxygen species, as is the case with longer wavelength UVA. The main target of reactive oxygen species is the purine base guanine. Modification of this base produces 8-hydroxyguanine which mispairs with adenine, leading to the formation of a T-A base pair from an original G-C base pair.

Single strand breaks.

Breaks in the sugar-phosphate backbone of DNA producing single strand breaks can be induced by both short and longer wavelength UV radiation. These types of DNA damage are rapidly repaired *in vitro* and the biological significance *in vivo* is unclear.

1.4.3 Repair of UV-induced DNA lesions.

DNA photoproducts will lead to skin cancer only if they persist long enough in basal cell keratinocytes to be fixed as mutations in subsequent rounds of cell division. Therefore accurate DNA repair mechanisms are critical for cell survival. The importance of accurate DNA replication has lead to the evolution of several sophisticated mechanisms for the repair of DNA damage (Friedberg, 1995).

Excision repair.

Excision repair removes areas of damaged DNA either as single bases or incorporated into small oligonucleotides. In both cases the repair process consists of four biochemically co-ordinated steps. These are:

1. Excision of the damaged base by a DNA glycosylase.
2. Incision of DNA at or near the damaged site by an endonuclease.
3. Resynthesis of DNA removed during repair using the complementary strand as a template, by the DNA polymerase.
4. Ligation of the newly synthesised DNA to the existing strand, by a DNA ligase.

Regardless of the mechanism by which damage is removed from DNA, excision repair generates single stranded gaps. Repair is not complete until the missing nucleotides are resynthesised by DNA polymerases and the repair gaps sealed by DNA ligase (Friedberg, 1995) (Fig 1.8).

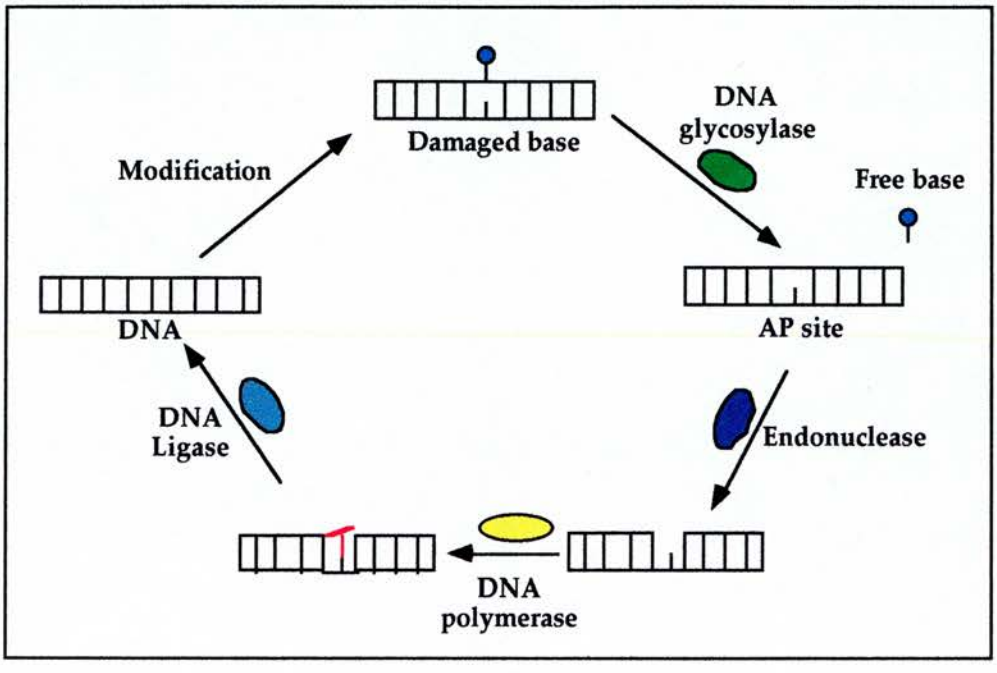
There are two sub pathways of nucleotide excision repair (NER). Transcription-coupled repair is responsible for the rapid repair of the transcribed strand of actively expressed genes and global genomic repair is for the slower, less efficient repair of the bulk of untranscribed DNA (Friedberg, 1996).

Post replication repair.

Post-replication repair pathways may be induced in response to DNA damage and allow cells to survive large damage burdens, often at the expense of increased mutation. Damage may still be present in the replicating DNA and such sites could block the progress of DNA polymerase synthesising the daughter strand. In order to avoid this, the cell activates one or more alternative pathways that allow DNA polymerase to bypass lesions in the parent strand, with consequent loss of information (gap) in the daughter strand, resulting in possible mutation. These gaps can be repaired in two different ways.

1. A gap filling mechanism that is based on repair synthesis (error prone repair)
2. Accurate post-replication repair when recombination is involved in filling the gap, called post-replicative recombinational repair. In this repair process the area of DNA damage in one double-stranded DNA molecule is replaced by the corresponding but undamaged sequence from the other.

Figure 1.8: Base excision repair.



The damaged base is excised by a DNA glycosylase enzyme, then the site is incised by an endonuclease, and the gap in the DNA is filled by a DNA polymerase enzyme and the new strand of DNA is rejoined by a DNA ligase enzyme.

1.4.4 Reactive Oxygen Species.

Reactive oxygen species, are thought to be involved in cancer, ageing and various inflammatory disorders. In normal circumstances ROS usually have a short half life, however they can react with DNA, proteins and unsaturated fatty acids (Engelhardt, 1999). A reaction of this type can result in DNA strand breaks, oxidative DNA damage, protein-protein and protein-DNA

cross-linking. In their reactions with lipids, ROS can produce lipid peroxides which persist within the cell and can lead to the initiation of free radical chain reactions in membranes with resulting membrane destruction.

In the body ROS are generated during respiration and during the inflammatory response. One exception is in the skin where the most important source of reactive oxygen species is UV-radiation. In the skin ROS can be formed directly or through (endogenous) photosensitisation reactions. During UV exposure in the skin a wide range of reactive species can be formed including singlet oxygen, superoxide anion, H_2O_2, nitric oxide, peroxynitrite and hydroxyl radicals. The ROS formed in the skin are thought to be involved in skin cancer, UV-induced immunesuppression and premature skin ageing.

Hydrogen peroxide is a dangerous molecule, due to it's small size and lack of charge it can diffuse a considerable distance and cross biological membranes. It is formed via the reaction of superoxide and hydrogen and this reaction is catalysed by superoxide dismutase (SOD).

$$2\,O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Superoxide anion — **Hydrogen peroxide**

The hydroxyl radical is also dangerous as it is a highly reactive and largely indiscriminate oxidant (Darr and Fridovich, 1994). The hydroxyl radical is formed when superoxide reacts with H_2O_2 in the presence of transition metals such as iron via the reaction:

$$H_2O_2 + O_2^- \xrightarrow{Fe^{2+} \longrightarrow Fe^{3+}} OH^{\bullet} + O_2 + OH^-$$

Hydroxyl radical

Singlet oxygen is also very reactive and will rapidly attack the double bonds found in unsaturated lipids. Superoxide anion is also very reactive and is

formed as a by product of respiration or directly by energy transfer via exposure to UV.

$$O_2 + e \longrightarrow O_2^-$$

Superoxide anion

Nitric oxide is generated by nitric oxide synthetases from L-arginine. Nitric oxide can react with superoxide to form peroxynitrite, which is a potent oxidant, which is capable of causing damage in a wide range of biological systems. It can nitrate proteins, attack lipids and cause DNA single strand breaks.

$$NO + O_2^- \longrightarrow OONO^-$$

Nitric oxide — **peroxynitrite**

Fortunately the skin possesses a wide range of antioxidant defence mechanisms to protect itself from UV-induced ROS (Fig 1.9). However the capacity of these antioxidant systems is not infinite and they can become overwhelmed by extensive exposure to UV radiation. The limited capacity of the antioxidant systems is supported by the fact that, following UV exposure most of the antioxidant systems in the skin are decreased. Therefore, following chronic exposure to UV, ROS can reach a damaging level.

One strategy to provide photoprotection would be to supplement or enhance the antioxidant systems.

1.4.5 Antioxidant systems in the skin.

Selenoproteins.

The family of glutathione peroxidases (GPXs) consist of four family members. They are all antioxidant selenoproteins. Selenoprotein P and the thioredoxin reductase are also antioxidant selenoproteins which will be covered in depth later in the chapter.

Catalase.

Catalase scavenges H_2O_2. Following exposure to UVA and UVB the level of catalase present in the skin is greatly reduced (Fuchs *et al*, 1989a and b; Hasegawa *et al*, 1992; Punnonen *et al*, 1991). The decrease in catalase is probably due to the irreversible oxidative damage of the enzyme. Catalase is thought to be a less important antioxidant enzyme than GPX, as catalase-deficient fibroblasts do not show decreased viability following exposure to UVB (Shindo and Hashimoto, 1995). It is interesting however to note that catalase can decrease the damage to other antioxidant enzymes during chronic UVB exposure (Shindo and Hashimoto, 1995). When added exogenously to tissue culture media, catalase can decrease the formation of sunburn cells in mouse skin explants following exposure to UVB (Miyachi *et al*, 1983).

Superoxide dismutase.

Superoxide dismutase catalyses the reduction of superoxide anions to the less reactive H_2O_2, which in turn can be converted via the Fenton reaction, in the presence of transition metals to produce the very reactive hydroxyl radical. Two forms of SOD exist, with different subcellular distributions. They are Cu/Zn SOD (Sherman *et al*, 1983) which resides in the cytoplasm and MnSOD (Ho and Crapo, 1988) which is expressed solely within the mitochondria. The level of SOD in the skin decreases following exposure to UVB (Fuchs *et al*, 1989a and b; Hasegawa *et al*, 1992; Punnonen *et al*, 1991; Miyachi *et al*, 1987). As seen with catalase the damage to SOD is probably a result of direct oxidative damage to the enzyme. When added exogenously to tissue culture media, SOD prevents the formation of sunburn cells (Miyachi *et al*, 1983). However the H_2O_2 formed by the action of SOD is itself damaging to cells, therefore to be protective any increase in SOD should be ideally accompanied with an increase in CAT or GPX (Yohn *et al*. 1991).

1.4.6 Non-enzymatic antioxidant systems.

Glutathione.

Glutathione is an endogenous tripeptide, it is the most abundant thiol-containing protein in the majority of tissues. Glutathione depletion makes cells more susceptible to cell killing by exposure to UV (Tyrrell and Pidoux, 1988). Glutathione can act directly as an antioxidant, it quenches free radicals by donating a hydrogen atom. Glutathione is also essential as it acts as the hydrogen donor for GPX and ascorbate. Therefore it is important in the maintenance of several antioxidant pathways.

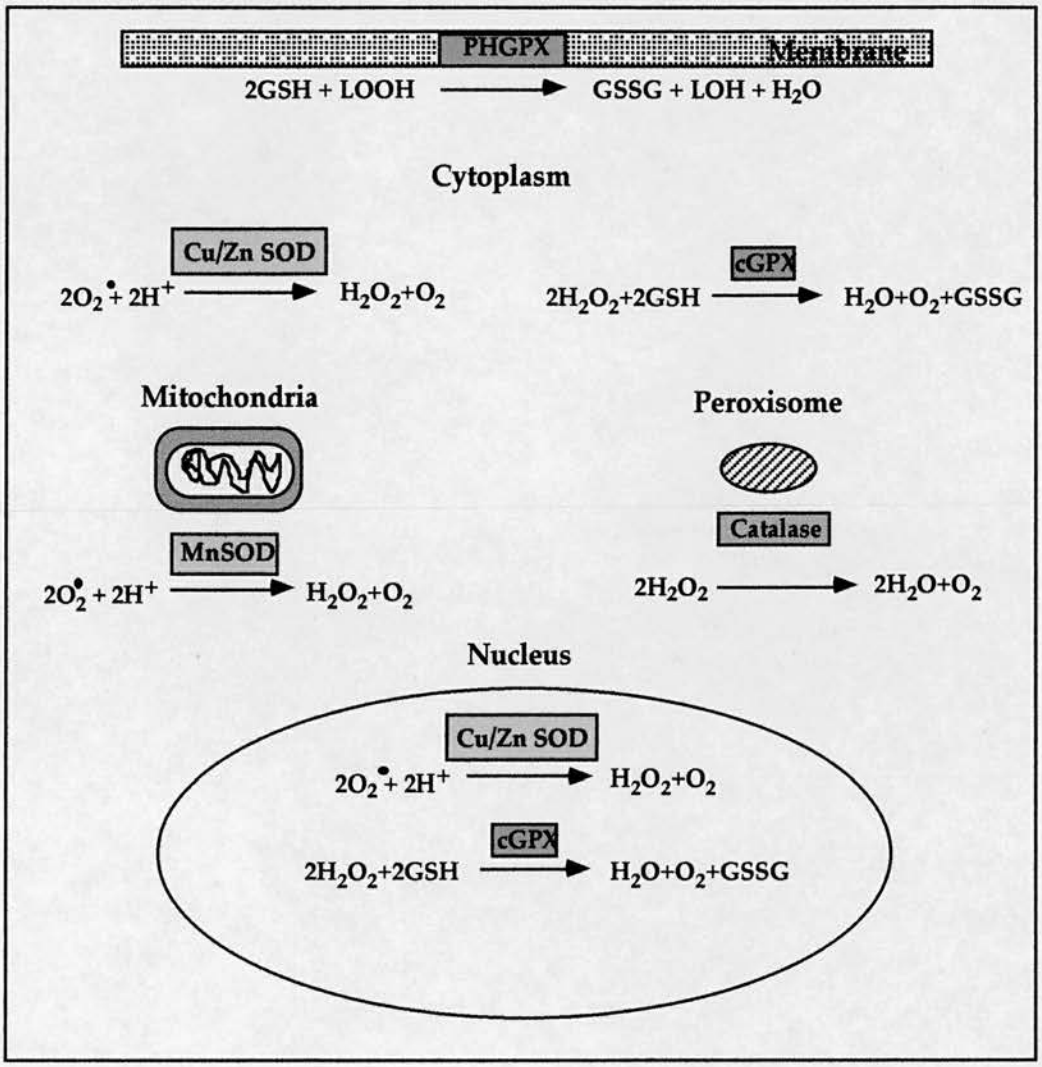
Glutathione is itself recycled by glutathione reductase with nicotinamide dinucleotide phosphate (NADPH) as a cofactor. The skin contains very high levels of glutathione reductase and it is estimated that it can successfully recycle all the glutathione present in the skin in less than one minute. This indicates how vital glutathione is in the skin (Conner and Wheeler, 1987). Cysteine derivatives such as N-acetylcysteine increase the intracellular levels of glutathione (Steenvoorden and van Henegouwen, 1997). In cultured fibroblasts N-acetylcysteine suppresses UVB-induction of p53 but not DNA single strand breaks (Renzing *et al*, 1996). It also prevents UVB induced immunesuppression in mice (van der Broeke and Beijersbergen, 1995). Glutathione derivatives such as glutathione-isopropylester decreases the sensitivity of cultured cells to UVB (Kobayashi *et al*, 1996a) and decrease photocarcinogenesis in mice (Kobayashi *et al*, 1996b).

α-tocopherol.

Alpha-tocopherol (Vitamin E) is a lipophillic endogenous antioxidant. It provides protection against UV-induced oxidative membrane damage (Burton and Traber, 1990). It functions as a chain breaking antioxidant in lipid peroxidation. Alpha-tocopherol is effective against UV-induced, immunesuppression (Yuen and Halliday, 1997; Gensler and Magdaleno, 1991; Clement-Lacroix *et al*, 1996), tumourigenesis (Gensler and Magdaleno, 1991), lipid peroxidation (Yuen and Halliday, 1997; Morliere *et al*, 1990), sunburn cell formation (LaRuche and Cesarini, 1991), depletion of epidermal

LCs (Yuen and Halliday, 1997), and oxidative DNA damage (Stewart *et al*, 1996).

Figure 1.9: Antioxidant systems in the skin.



Antioxidant systems in the skin, phospholipid glutathione peroxidase (PHGPX), cytoplasmic glutathione peroxidase (cGPX), lipid peroxides (LOOH), glutathione (GSH).

1.5 Effects of UV radiation on the skin immune system.

It is thought that the skin immune system is important in the control of certain tumours by performing an immune surveillance role, monitoring for changes associated with cellular transformation. In order for the

transformed cell to evade the immune system, the immune system itself would have to be weakened in some way. The phenomena of UV-induced modulation of tumour immunity was first reported by Kripke and co-workers in 1977. They discovered that UV-induced murine skin tumours are highly immunogenic and are therefore rejected upon inoculation into naive syngenic hosts. However the tumours grew progressively if the recipient had been UV-irradiated prior to receiving the tumour, with subcarcinogenic doses of UV radiation (Kripke and Fisher, 1976; Fisher and Kripke, 1977; Kripke, 1994). Further evidence that immunesuppression plays a role in skin carcinogenesis, comes from data on renal allograft recipients receiving immunosuppressive drugs. These individuals have a highly increased risk of developing SCCs, which are generally found on sun-exposed sites (Hartevelt *et al*, 1990). Therefore UVB appears to have a role in both tumour initiation by causing DNA damage and as a tumour promoter by inhibiting tumour surveillance and hence can be described as a complete carcinogen (Brash *et al*, 1996).

It has been suggested that DNA damage can itself induce immune suppression via the production of immunoregulatory cytokines (Kripke *et al*, 1992). Application of the DNA repair enzyme T4 endonuclease V encapsulated in liposomes, onto UV-irradiated skin of mice prevents the loss of LC from the skin and reduces local immune suppression (Wolf *et al*, 1995). It has also been demonstrated that DNA damage in keratinocytes induces the release of immunomodulatory cytokines (O'Connor *et al*, 1996), resulting in immune suppression respectively.

Although cytokines are not expressed constitutively by keratinocytes *in vivo* they can be induced by a variety of stimuli. Furthermore, expression of cell surface molecules such as, MHC class II antigens or ICAM-1 are induced on keratinocytes in inflammatory reactions of the skin (Barker *et al*, 1990). MHC class II+ keratinocytes can also present bacterial superantigens, *Mycobacterium leprae* and herpes simplex virus antigens to T cells *in vitro* (Nickoloff *et al*, 1994; Cunningham *et al*, 1989), thus emphasising the potential role of keratinocytes in regulating the skin immune system.

Many cytokines are released from keratinocytes following exposure to UVB these include, IL-1α which is involved in the pro-inflammatory reaction and

induces fever, inflammation, erythema and the release of other cytokines from both keratinocytes and fibroblasts (Sauder *et al*, 1982). Another pro-inflammatory cytokine which is released following exposure to UV is IL-6, it can induce acute phase proteins, fever and keratinocyte proliferation (DeVos *et al*, 1994). IL-8 is also induced following exposure to UV radiation, it functions as a chemoattractant for neutrophils (Kondo *et al*, 1993).

Other cytokines which are induced following exposure to UV have immunosuppressive functions. IL-10; has been shown to be immunosuppressive and can suppress inflammatory cytokine synthesis. IL-12 is important in mediating cell-mediated immune responses (reviewed in Duthie *et al*, 1999).

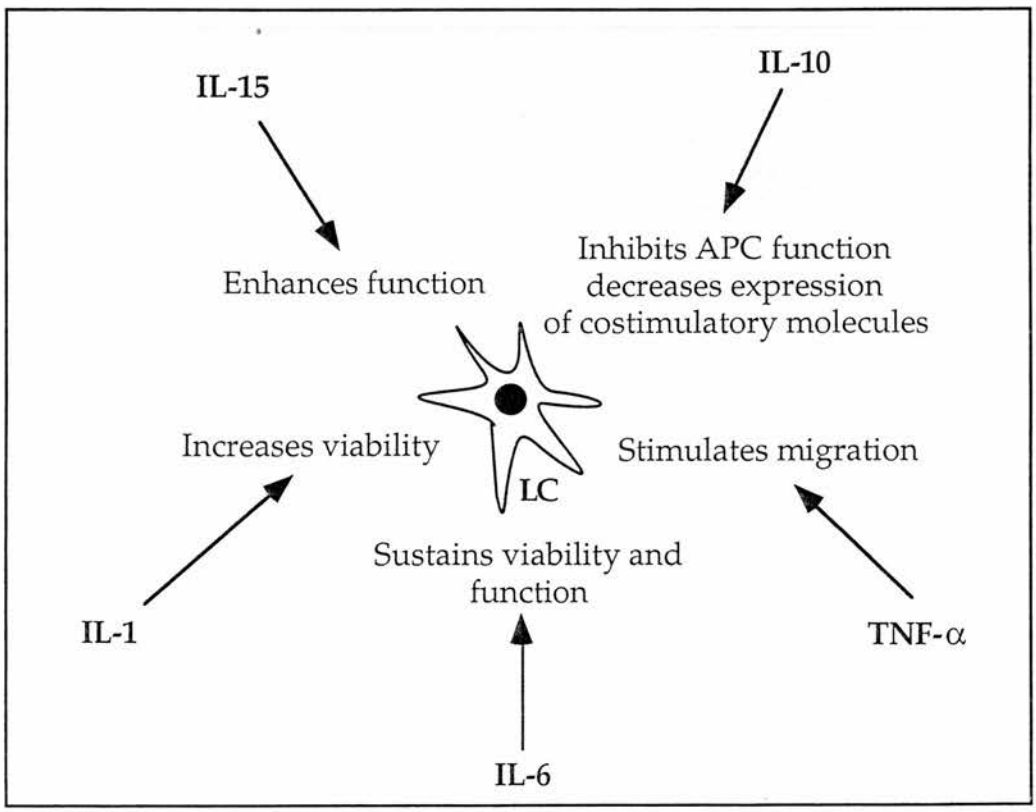
TNF-α is also upregulated following exposure of the skin to UV, it has been proposed to initiate the movement of LCs from the skin to the draining lymph nodes (Cumberbatch and Kimber, 1995). IL-1β has also been implicated in the migration of LCs to the draining lymph nodes (Cumberbatch *et al*, 1997). It is thought that IL-1β is mainly LC-derived and can upregulate epidermal TNF-α (Enk *et al*, 1993). The effects of cytokines on LCs can be seen in Fig 1.10. TNF-α as well as being involved in LC migration is also a potent inflammatory cytokine and induces prostaglandin and collagenase release. Exposure to UV has other effects on LCs: they can loose their dendrites, show a decreased ability to present antigens and if badly damaged following exposure can undergo apoptosis (Rattis *et al*, 1998) (Fig 1.11). All of these effects lead to a decreased immune response to antigens.

The main functions of these UV-induced cytokines will be discussed further in Chapter 6 and 7, however some of the functions of the UV-regulated cytokines are illustrated in Fig 1.12.

One of the most widely used methods for studying immune suppression in mice is the contact hypersensitivity method (reviewed in Beissert and Schwartz, 1999). A sensitising chemical is applied to the skin, the antigen of choice is taken up and processed by the LCs, which migrate to the draining lymph nodes, were they present the antigen to T cells. The T cells proliferate and differentiate into an effector population. When the antigen is applied a

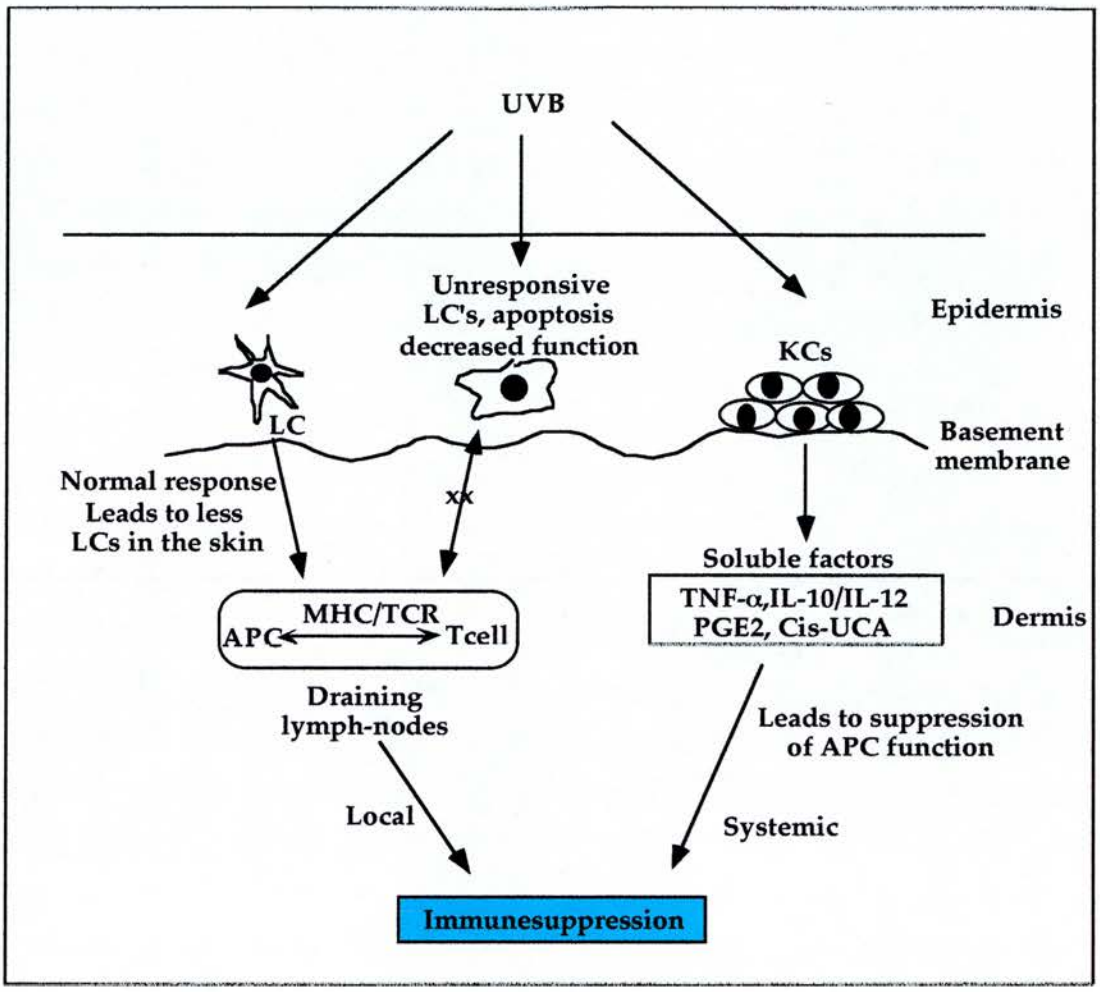
second time either to the same site or a different one, an immune response occurs to clear the antigen and tissue swelling occurs. The swelling can be measured, if the site of application is the ears, ear swelling can be measured. If the mice are irradiated prior to the first exposure of antigen then the observed immune response is greatly decreased, this is accounted for by immunosuppression caused by UVB (Moodycliffe, 1994, Toews *et al*, 1980). The use of contact hypersensitivity is a versatile method for studying the immunosuppression induced by UV. TNF-α is thought to be involved in local immunosuppression by UV, perhaps by inducing LC to migrate from the epidermis (Moodycliffe *et al*, 1994,). The cytokine IL-10 is an important mediator of systemic immune suppression following exposure to UV (Rivas and Ullrich, 1992).

Figure 1.10: Effects of UV-inducible cytokines on LCs.



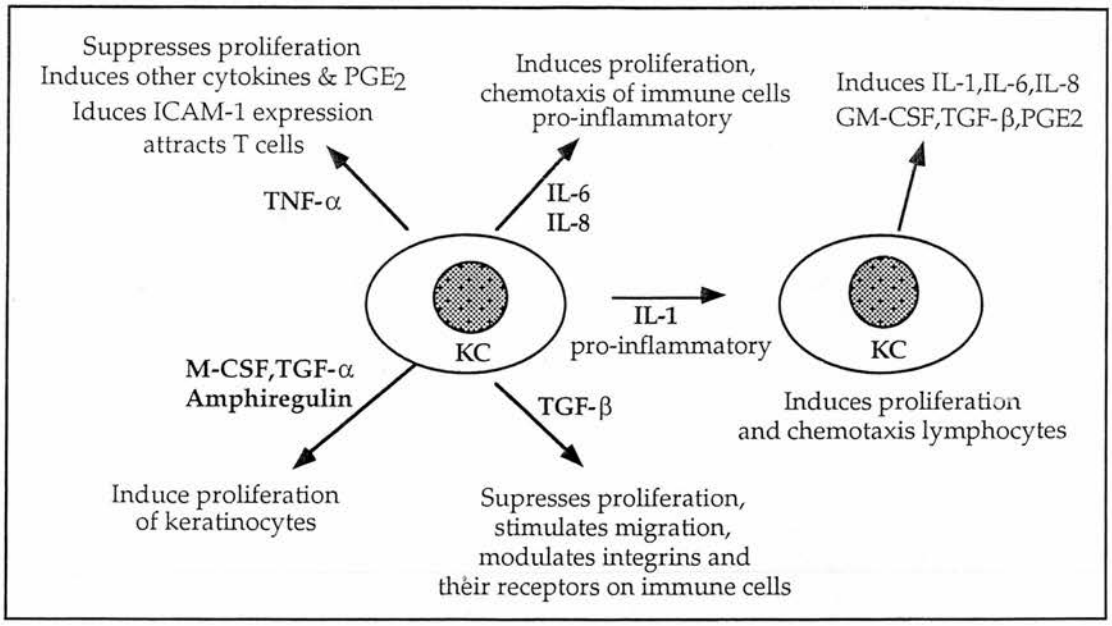
Effects of keratinocyte cytokines on LCs (adapted from Kondo, 1999).

Figure 1.11: Effect of UVB on LCs in the skin.



LCs once irradiated; migrate from the epidermis, thus leaving a reduced LC population to provide antigen presentation. Also LCs can be damaged by UV and show a decreased ability to present antigens. Antigen presenting cells (APCs) present antigens to T cells in the draining lymph-nodes. Finally soluble factors produced by keratinocytes (KCs) following exposure to UVB can lead to systemic immunesuppression (adapted from Meunier, 1999).

Figure 1.12: Cytokines released from keratinocytes following exposure to UV radiation.



Autocrine and paracrine effects of some the cytokines released from keratinocytes (KC) following exposure to UV radiation (adapted from Kondo, 1999).

1.6 Transcription factor activation by UV radiation.

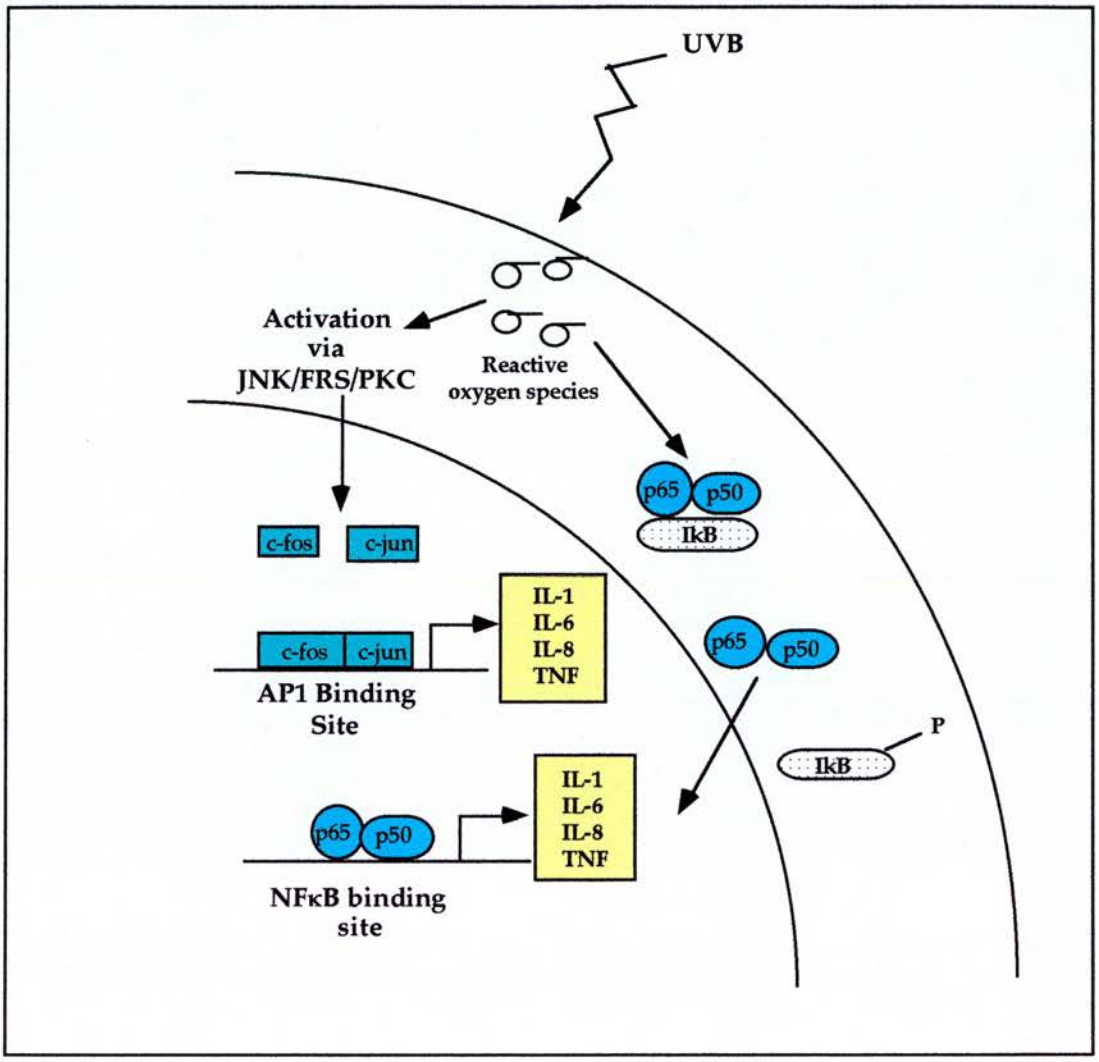
Many transcription factors are also activated by exposure to UV radiation. The c-jun and c-fos proto-oncogenes are both rapidly induced following exposure to UV. The jun and fos gene products form heterodimers called activator protein-1 (AP-1), a transcriptional activator that binds to either the TPA response element or the AP-1 sites on genes and subsequently up regulates many other UV inducible genes (reviewed by Foletta *et al*, 1998) (Fig 1.13). AP-1 activates many genes including; IL-4, -5, -3, -6, -8, -1β, TNF-α.

Another transcription factor nuclear factor kappa B (NFκB) is found in the majority of cell types. It is composed of a family of five different proteins, which form dimers in the cytoplasm of cells, examples of this family are p50

and p65. Within the cytosol, NFκB is kept inactive by binding to the inhibitory complex IκB. On receiving an activation signal NFκB dissociates from IκB and moves into the nucleus, where it can activate gene transcription of many genes including; IL-1α and β, IL-2, -3, -6, - 8, -12 and TNF-α to name a few (reviewed in May and Ghosh, 1998; Ghosh *et al*, 1998) (Fig 1.14). NFκB can also be activated in enucleated cells, this suggests that the activation signal occurs at the cell membrane.

Other signal transduction proteins which are activated by UV include: Src tyrosine kinases (Devary *et al*, 1992), Ras (a small GTP binding protein) (Engelberg *et al*, 1994), JNK-1 (Derijard *et al*, 1994), GADD and MAP kinases. Exposure to UV can also lead to the activation of phospholipase A_2 (Hawk *et al*, 1983; Hanson and Deleo, 1989, 1990). Phospholipase A_2 activation leads to the release of archadondic acid from membrane phospholipids and plays an essential role in the generation of inflammatory intermediates such as, leukotrienes and prostaglandins (Tyrrell, 1996). Furthermore, there is considerable evidence that UVB radiation can lead to prostaglandin E2 synthesis via pathways which can be inhibited by antioxidants (Miller *et al*, 1994).

Figure 1.13: Transcriptional activation following exposure to UVB.



AP-1 and NFκB activation following exposure to UVB. c-fos and c-jun are activated and form a dimer which can then bind DNA and activate the transcription of other genes. IκB is phosphorylated, IκB dissociates and NFκB translocates into the nucleus where it can bind DNA.

1.7 Selenium.

1.7.1 Introduction.

The trace element Selenium (Se) was discovered by Jacob Berzelius in 1817 and was named after Selene the Greek goddess of the moon. It is in group VI of the periodic table, below sulphur and has an atomic weight of 78.96. It shares many properties with sulphur, they are both of similar atomic size, bond energy and electron affinities. It is a metalloid and has semi-conducting properties. Se exists naturally in a range of oxidation states as volatile species or analogues of organic sulphur compounds, the properties of which are described in Table 1.1.

Table 1.1: Oxidative states in which Se is found (adapted from Foster and Sumar, 1997).

Form	Occurrence	Properties
Selenate (SeO_4)	Alkaline waters, soils, plants, food chain	Soluble, stable in alkaline/oxidising conditions.
Selenite (SeO_3)	As above	Readily oxidised by alkali pH, oxygen. Readily reduced by SO_2, ascorbic acid. Binds to iron and aluminium in soil, forms anhydride Se oxides.
Selenide (H_2Se)	Heavy metal selenides in minerals, unavailable to plants	Insoluble in soils
Elemental Se (Se)	Unavailable to plants	Forms complex bonds with Br and Cl. Stable insoluble Allotrophic forms at room temperature.

Selenium is widely used in industry in photocopying machines, light meters, it is used in stainless steel production, vulcanising rubber and it is added to many anti-dandruff shampoos.

At low levels it is an essential element necessary for the growth of animals and humans. However at higher concentrations it can be toxic. Selenium's nutritional value was first discovered in 1957 when it was found to prevent liver necrosis in vitamin E-deficient rats (Schwartz and Foltz, 1957). In 1973 the role of Se in the antioxidant enzyme glutathione peroxidase (GPX) was discovered (Rotruck *et al*, 1973).

Se is mainly ingested as selenoamino acids (selenomethionine and selenocysteine) from plants and animals (Spallholz, 1994) or as methylated/non-methlylated Se (Table 1.2). Selenoamino acids are then transported across the gut via amino acid transport proteins. Most forms of Se are then converted to hydrogen selenide in the body then incorporated in to selenoproteins (Fig 1.14). Excess Se can be methylated to mono, di or trimethyl, Se. Trimethly Se is excreted by the kidneys through the urine, and the dimethyl form is exhaled.

In Se supplementation trials, both organic and inorganic forms of Se are rapidly absorbed. However organic forms are retained for longer, and reach higher concentrations. This maybe due to the incorporation of selenomethionine into non-specific cellular proteins, instead of methionine (Waschulewski and Sunde, 1988). Inorganic forms of Se increase the level of platelet GPX more rapidly than organic forms (Neve, 1995; Brown *et al*, 2000).

The bioavailability of Se can be dependent on the levels of methionine in the diet, total protein content, restricted diet intake, presence of heavy metals and the levels of some sulphur compounds.

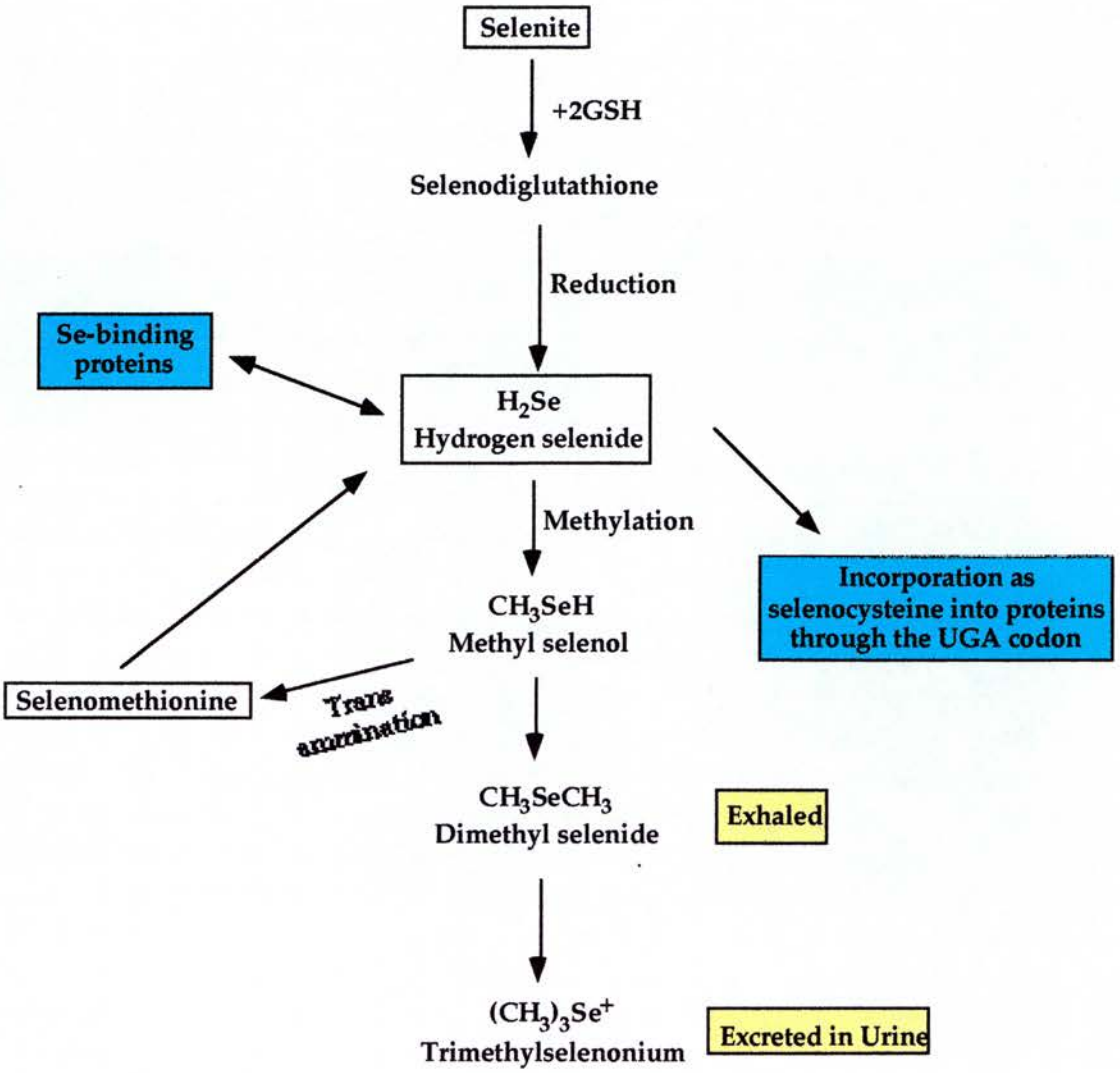
Se occurs in all human tissues especially the liver and kidney at an average level of about 0.2 μg/g. It can be either be incorporated covalently into selenoproteins whose active sites contain Se as selenocysteine. Alternatively Se may be loosely bound into Se-binding proteins, or incorporated non-specifically as selenomethionine instead of methionine.

Table 1.2: Most common Se compounds (adapted from Foster and Sumar, 1997).

Form	Dietary Se compounds	Characteristics
Inorganic	Sodium selenite, selenious acid	Readily absorbed /utilised by humans, Used for Se supplementation in Se deficient areas
	Sodium selenate, selenic acid	More stable
Organic	Selenomethionine	Predominant form of Se, stable, readily absorbed, Used for Se supplementation
	Selenocysteine	Bioactive form occurring in selenoproteins
Synthetic	Selenocystamine anti - infective phenylselenourea - antifungal	Se is incorporated into potential medicinal agents

Se status is usually assessed by measurement of Se in whole blood or it's fractions. GPX activity can also be measured in erythrocytes. However GPX expression in erythrocytes reaches a plateau and so it is of little use for assessing high Se intake. In practice measurement of plasma Se is the most commonly used method to assess Se status (Neve, 1995). Se is mainly excreted in urine, so urine measurements can be of use in the assessment of Se status. Many studies have used nail and hair samples as a measure of long term Se status, since the amount of Se in the nails increases after Se supplementation.

Figure 1.14: Selenium metabolism.



(adapted from Ip and Ganther, 1990).

The levels of Se in foodstuffs varies considerably depending on the concentration of Se found in the underlying soil. Regions of the world with very low soil Se (<0.05 ppm) include, Denmark, Eastern Finland, North East and Southern central China. Less severe deficient areas have been reported in Western Australia, New Zealand and the USA. The average daily intake of Se also varies depending on the Se levels in general food products. In the USA the daily average intake is 132 μg/day, whereas in Britain it ranges from 34 μg- 43.7 μg/day and in Scotland is about 48.7 μg. The average daily intake of Se in the UK has been decreasing over the last 30 years and in the 1970s it was around 60 μg/day (Rayman, 1997). The recommended daily

intake in the USA is 70 μg/day (Levander, 1997). This level is aimed to be sufficient to increase the level of GPX in the plasma to plateau levels. However this may be an underestimate of how much Se is required for optimal health and for other selenoproteins to reach their maximal expression (Neve, 1995). The Food and Nutrition Board of the US National Research Council estimate the safe and adequate Se intake for adults is 50-400 μg/day (Levander, 1983). The highest levels of Se in the diet are found in Brazil nuts (124 μg/100g), kidney (146 μg/100g) and crab meat (84 μg/100g), whereas levels in dairy products were lower (below 2 μg/100g).

1.8 Selenium deficiency.

Se deficiency in cattle and livestock is associated with white muscle disease (Oldfield, 1987). It is a localised myopathy associated with depigmentation and calcification. Se deficiency in humans (<11 μg/day) is associated with Keshan disease, a myocardiopathy associated with children and young adults. In Heilongjiang province in China the disease was rampant in the 1930-1960s. During the 1960s task groups were set up to study the problem and Se deficiency was found to be a major factor. The disease occurred in focal patches in a belt from the Northeast to the Southeast of China. The disease primarily struck children under five. One factor which suggests that Se deficiency was not the only causal factor, was the seasonal occurrence of the disease. The disease peaked in the Northeast in winter and in the Southeast in summer. Features of the disease include chronic cardiac insufficiency, heart enlargement, gallop rhythm, arythmia, multifocal necrosis and fibrous replacement of the myocardium. Since 1974 table salt has been supplemented with selenite in areas of China with low soil Se levels and the disease has virtually been eradicated, with only 45 cases reported in 1990 in the whole of China (Yang *et al*, 1984). It is thought that low Se intake and oxidative stress are causal factors in this disease. Lack of other antioxidant nutrients are also thought to play a role as well. Exposure to coxsackie virus is thought to be a factor in the pathogenesis of Keshan disease; the virus causes more damage to Se-deficient heart tissue. It has also been demonstrated that the non-virulent form of the virus, can become virulent when passaged through a Se-deficient host. The mechanism for this is not presently known, however it has been hypothesised that the viral RNA may undergo free-radical induced alteration in the Se-deficient host

(Levander and Beck, 1997). Indeed the nucleotide sequence of the non-virulent virus closely resembles the virulent form following passage through a Se-deficient host (Beck and Levander, 1998). There have been further suggestions that Se deficiency may exacerbate other viral infections including; influenza, hepatitis and human immunodeficiency virus (Baum *et al*, 1997).

Another disease found in areas with low levels of Se in the soil is Kasin-Beck disease (osteoarthritis deformans endemica) or enlarged joint disease. It occurs in northern China, North Korea and eastern Siberia. It affects mainly children aged 5-13 years. It starts as limb weakness, stiffness of the joints, swelling of the joints and progresses to joint enlargement and dysfunction by 30 years of age (reviewed in Levander, 1987). Again the disease can be prevented by Se supplementation.

Se deficiency has been linked to an increased risk of artherosclerosis and other forms of cardiovascular disease. This maybe due to increased levels of free radicals, oxidised low density lipoprotein and lipid peroxidation. Selenium deficiency also leads to abnormal sperm structure and reduced fertility (Becvana and Bahuguna, 1994). Children fed intravenously long term also show signs of Se deficiency including hair loss, myopathy and macrocytosis.

Epidemiological studies have suggested that there is a higher incidence of colon, stomach, bladder, ovarian, pancreas, oesophagus, breast and other types of cancer in areas with low Se in the soil (Shamberger and Willis, 1971; Schrauzer *et al*, 1977a and b; Yu *et al*, 1991; Knekt *et al*, 1990).

1.9 Selenium supplementation trials.

Following publication of experimental evidence in animal models which suggested that Se supplementation decreased tumourigenesis, several human trials have been carried out. The largest trial was carried out by Clark *et al*, 1996 who reported a lower incidence of colorectal, lung, and prostate cancer rates in patients supplemented with Se. The trial was carried in Se-poor regions of the USA. In the study 1300 patients with a history of at least 2 skin cancers, were given either placebo tablets or Se tablets in the

form of 200 μg Se/day of a Se enriched brewers yeast. The trial was fully randomised and was carried out over a 10 year period. During the trial the average plasma Se level had increased by 67% with no signs of toxicity. They found a 37% reduction in the incidence of total cancers, a 50% decrease in cancer mortalities and a 17% decrease in total deaths. There were significant reduction in the numbers of patients with prostate (63%), colorectal (58%), and lung cancers (46%) in the Se supplemented group (Clark *et al*, 1996). They found no effect on the levels of skin cancers. An earlier trial in China had shown a 35% decrease in hepatoma incidence in patients receiving Se supplements (Yu *et al*, 1990).

Another large scale supplementation trial was started in 1994 in France (SUVIMAX trial). The trial is to be carried out for 8 years on 15,000 people to investigate whether Se and Zinc supplementation reduces the incidence of cancer and heart disease. The PRECISE trial is the most recent Se supplementation trial, it is about to begin in the UK, Denmark, Sweden, Finland and the USA. There will eventually be 40,000 patients recruited and they will receive either 100, 200 or 300 μg of Se a day, the trial will last for 5 years and will study the mortality rate in all of the groups.

Se supplementation has been suggested for many medical conditions, including, cataracts, malaria, alopecia areata, muscular dystrophy, and male infertility. It has also been suggested that Se supplementation can improve arthritis (Tarp *et al*, 1985, 1995).

Several theories have been offered to explain the protective effects of Se in human health. They include prevention of carcinogen-induced oxidative damage, alterations in carcinogen metabolism and selective toxicity to rapidly dividing tumourogenic cells. It has been demonstrated that selenite and selenomethionine at high levels (μM) protect against tumourogenesis. A whole range of new synthetic forms of Se compounds are presently being produced and tested for their anti-tumourogenesis effects. One such compound is 1,4-pheylenebis(methylene)selenocyanate (p-XSC). This compound has low toxicity and can inhibit tumourogenesis in mammary glands, the colon and the lungs in experimental animals (El-Bayoumy *et al*, 1995).

The longest wide scale Se supplementation programme is running in Finland, where a program of nation-wide Se supplementation (as sodium selenate) in fertilisers has been ongoing since 1984. It was decided to supplement fertilisers in Finland with Se due to the very low levels of Se in the soil, which lead to food products containing low levels of Se. The plasma Se levels in the Finnish population were very low in the 1970s and there was a high prevalence of heart disease and cancer. The Se content of plasma in Finland was between 0.63-0.76 μM in 1970 and has now risen to 1.40 μM (Varo *et al*, 1993).

1.10 Selenium toxicity.

Although Se is an essential element at high levels it can become toxic. Se toxicity is dependent on the type of Se, and species of animal. In humans the symptoms are garlicky breath odour which is due to exhaled dimethly selenide, brittle nails, discolouration of the nails and hair loss (Bedwal *et al*, 1993). Some parts of China have very high levels of Se in the soil, where daily intake can be as high as 900 μg/day. In Britain the recommended maximum safe amount of Se is 450 μg/day. Selenosis in animals can result in hoof rot, blind staggers due to weakness of the forelimbs, alkali disease and alopecia. Certain plants from the Astragalus genus can be toxic to cattle due to their ability to accumulate Se.

The toxicity of high levels of Se, is thought to be due to its prooxidant ability to catalyse the oxidation of thiols and simultaneously generate superoxide (Spallholz, 1994). Cytotoxic Se compounds include; selenite and selenocysteine, which can generate superoxide and lead to an increase in oxidative DNA damage in the cell leading to apoptosis (Stewart *et al*, 1999). Selenite can also react with glutathione to produce selenodiglutathione, a very reactive compound, which has antiproliferic and apoptosis inducing properties (Wu *et al*, 1995a). Selenomethionine is not cytotoxic to cells even at high concentrations as it is a non-redoxing, Se compound which does not produce superoxide (Stewart *et al*, 1999). High levels of cytotoxic Se compounds have been shown to be anticarcinogenic in some animal models (Medina, 1986 and 1988; Ip, 1986). The mechanism of this anticarcinogenic effect is unknown but many theories have been suggested. One such hypothesis is that the high levels of some Se compounds, for example

selenite, inhibits the growth of tumour cells. Another possibility is that methylated Se derivatives inhibit crucial redox enzymes or can metabolise chemical carcinogens (Lanfear *et al*, 1994). Selenite and selenodiglutathione have also both been shown to inhibit ribonucleotide reductase activity at high levels (10 μM), inhibition of *de novo* synthesis of deoxyribonucleotides prevents cell cycle progression and leads to an accumulation of cells in S phase. This accumulation of cells in S phase does occur at high levels of selenite (Spyrou *et al*, 1996).

1.11 Selenoproteins.

It is now recognised that Se exerts many of its actions through the expression of a number of selenoproteins, in which Se is covalently linked within the protein, as selenocysteine (Cone *et al*, 1976). Replacing selenocysteine with cysteine results in a sharp decline in activity of selenoproteins (Axley *et al*, 1991; Rocher *et al*, 1992). Thus the effective function of these enzymes depends on insertion of the selenocysteine residue. The Se atom of selenocysteine is more reductive than the sulphur atom of cysteine, in part because it is fully ionised at physiological pH in contrast to cysteine containing sulphur. At least 30 selenoproteins have been identified by SDS-polyacrylamide electrophoresis of ^{75}Se-labelled tissue. However only approximately fourteen have been characterised.

1.11.1 Selenoprotein synthesis.

The synthesis of selenoproteins in prokaryotes is dependent on the products of 4 genes Sel A, Sel B, Sel C, and Sel D (Burk and Hill, 1993; Burk, 1991). The Sel C gene product is a unique form of transfer RNA (tRNA) which acts as a serine/phosphoserine carrier and recognises the UGA stop codon for selenocysteine insertion. The Sel A gene codes for a pyridoxal phosphate -containing, selenocysteine synthetase enzyme which converts the serine on the tRNA, specified by the Sel C product to selenocysteine in the presence of the product from the Sel D protein. The Sel D product requires ATP and magnesium for it's activity, and is thought to produce phosphoselenoate. The serine is first phosphorylated to phosphoserine before the phosphate group is substituted with Se, as phosphoselenoate to produce selenocysteine tRNA. The Sel B product is required for co-translation and insertion of

selenocysteine into proteins and is similar to the elongation factor-Tu which transports amino acid tRNAs to the ribosome.

In addition to a UGA codon a specific 3'-untranslated segment referred to as the selenocysteine insertion sequence (SECIS), needs to be present in the mRNA to allow the UGA codon to read for selenocysteine and not for termination of translation (Berry *et al*, 1997; Low and Berry, 1996). The sequences are variable for different selenoproteins, however they are thought to serve a common purpose. The insertion sequences create a stem loop structure, which allows recognition of the UGA codon as a selenocysteine instead of a termination codon. In prokaryotes the SECIS is adjacent to the UGA codon, however in eukaryotes it is located in the 3' untranslated region (Stadtman, 1996). Parts of the mechanism for insertion of selenocysteine into selenoproteins has been characterised and it appears to be similar to that in prokaryotes. A specific tRNA has been isolated, SECIS elements are present and elongation factors have been isolated in the mammalian system.

1.11.2 Hierarchy of selenoproteins.

There is an important hierarchy of Se supply to tissues during Se deficiency, the thyroid, brain, skin and lymph nodes retain the element, whilst it is rapidly lost from liver, kidneys and muscle (Arthur *et al*, 1993; Beckett *et al*, 1993; Bermano *et al*, 1995; Calomme *et al*, 1995; Thompson *et al*, 1995). In addition there is a an important hierarchy of Se supply to different selenoenzymes within tissues, such that loss of expression of cyGPX occurs before most other selenoproteins..

1.11.3 Selenoprotein P.

Selenoprotein P is an extracellular glycoprotein, that makes up one third of the total Se in human plasma and it is thought to have an antioxidant defence role (Burk *et al*, 1995). Selenoprotein P can protect the liver from diquat-induced liver necrosis in mice (Burk *et al*, 1991). Selenoprotein P is secreted into the plasma by the liver (Burk and Hill, 1999). There is evidence to suggest that one possible function of selenoprotein P is to protect endothelial cells from oxidative damage (Burk *et al*, 1997). The protein has a

molecular mass of 55 kDa and has been shown to contain 10 in-frame selenocysteine residues (Hill *et al*, 1991). It also contains two SECIS motifs in the 3' untranslated region. The protein can exists as several isoforms, one isoform being formed by termination of translation after insertion of 2 selenocysteines (45 kDa). Another isoform is formed following termination after insertion of all 10 selenocysteine (55 kDa) (Chittum *et al*, 1996). The expression of the protein is modulated by Se status (Yang *et al*, 1989).

1.11.4 Iodothyronine 5-deiodinases.

The family of iodothyronine deiodinases (Types I, II and III) are all selenoenzymes. Type 1 iodothyronine 5-deiodinase (IDI) is responsible for 5' monodeiodination and thus conversion of the prohormone thyroxine to the active hormone triiodothyronine. Type -I IDI can also catalyse 5-monoidination and thus convert thyroxine to the inactive isomer of reverse triiodothyronine. The enzyme is a homodimer and each 27 kDa subunit contains one selenocysteine (Arthur *et al*, 1990; Berry *et al*, 1991). Type I-IDI is found in high levels in the liver, kidney, thyroid and muscles, it carries out local 5 and 5' deiodination. During Se deficiency the levels of thyroidal IDI are conserved, whereas hepatic IDI levels decrease (Arthur *et al*, 1993; Beckett *et al*, 1993). Type II-IDI in comparison to type I-IDI carries out only 5' deiodination, whilst Type III-IDI catalyses only 5-deiodination (St. Germain and Galton, 1997).

1.11.5 Selenoprotein W.

Selenoprotein W was first found as a component of skeletal muscle protein (Vendeland*et al*, 1993), loss of selenoprotein W expression occurs prior to the onset of white muscle disease in Se deficient cattle. Four isoforms have been found, each having one Se atom per molecule. The molecular weights are 9550, 9596, 9858 and 9898 daltons. Selenoprotein W expression is found in greatest quantities in the brain, muscle, testis and spleen. It may have a redox function as it has been isolated with glutathione (Burk and Hill, 1999).

1.11.6 Novel Selenoproteins.

Recently a novel 15 kDa selenoprotein has been discovered which is found at high levels in the prostate (Gladyshev *et al*, 1998).

1.11.7 Selenium-binding proteins.

These proteins covalently bind Se in an unknown form which is not selenocysteine. The biological significance of the Se is unknown and the activity of these proteins are not regulated by Se status (Wu *et al*, 1995b). Proteins of this type include protein disulphide isomerase, which is involved in protein folding (Sinha *et al*, 1993), a 15 kDa protein called fatty acid binding protein (Bansal *et al*, 1989), and a 56 kDa protein (SP56) which is closely related to acetaminophen-binding protein (Lanfear *et al*, 1993).

1.11.8 Glutathione peroxidases.

Several forms of GPX have been discovered these include cytoplasmic GPX (cGPX), phospholipid hydroperoxide GPX (PHGPX) (Ursini *et al*, 1985), plasma or extracellular GPX (EGPX) (Takahashi and Cohen, 1986) and gastrointestinal (gGPX) (Wingler *et al*, 1999)

The family of GPXs detoxify a wide range of lipid peroxides and hydrogen peroxide to produce water and the corresponding alcohol in the presence of reduced glutathione (Roetruck *et al*, 1973; reviewed in Brigelius-Flohe, 1999).

Cytoplasmic GPX.

The first functional selenoprotein to be discovered was cytoplasmic GPX, it compromises of four identical subunits, each with a molecular weight of 21-26 kDa (Gladyshev and Hatfield, 1999) and containing one selenocysteine residue (Roetruck *et al*, 1973). Cytoplasmic GPX may also acts as a reservoir for Se (Burk, 1991; Sunde, 1990). Cytoplasmic GPX is expressed by most tissues in the body. The importance of cGPX is unclear as mice which are deficient in cGPX develop normally and appear to cope with hypoxia (Ho *et al*, 1997). However other groups have reported that cGPX knockout mice are more susceptible to oxidative stress (Cheng *et al*, 1999; Fu *et al*, 1999).

The mRNA for cGPX has a guanine base immediately following the UGA coding for insertion of Se into the protein (Low and Berry, 1996). This terminator purine has been proposed to favour termination when selenocysteine-tRNA is limiting (McGaughan *et al*, 1995; Allan *et al*, 1999). It has also been proposed that cysteine substitutes for selenocysteine and is incorporated into the protein during periods of Se deprivation. The cysteine containing protein is less active than the Se containing form of the protein (Berggren *et al*, 1997). Se depletion in some cell types leads to a decreased half-life of cGPX mRNA but not PHGPX mRNA (Bermano *et al*, 1995; Bermano *et al*, 1996a).

Phospholipid hydroperoxide GPX.

PHGPX is monomeric and has one Se atom per molecule, it has a molecular weight of 20-23 kDa (Ursini *et al*, 1985; Schuckelt *et al*, 1991). It occurs in plasma, breast milk, the heart, liver and lung levage. The protein PHGPX has been suggested to have an antioxidant activity in extracellular fluids. The protein may have a role in protection against cardiomyopathy and Keshan disease. Unlike cGPX, PHGPX can metabolise phospholipid hydroperoxides, lipid peroxides and low density lipoprotein and is believed to prevent damage to membranes (Ursini *et al*, 1985; Maiorino *et al*, 1991). PHGPX would appear to have a role in modulating leukotriene biosynthesis (Weitzel and Wendel, 1993; Schnurr *et al*, 1996; Imai *et al*, 1998; Huang *et al*, 1998). During Se depletion the activity of PHGPX is decreased, however the rate of this decrease in activity is significantly slower than that for cGPX (Weitzel *et al*, 1990). When PHGPX is transfected into cells it provides protection from lipid hydroperoxide-mediated cell injury (Sun *et al*, 1997). The expression of the protein and it's activity increase on addition of Se.

PHGPX also plays an important role in spermatogenesis. The protein for PHGPX is present in spermatids as a soluble enzyme exhibiting high activity, however during spermatogenesis the PHGPX polymerises. The PHGPX becomes an inactive cross-linked insoluble protein which is bound to the mitochrondrial capsule (Ursini *et al*, 1999).

Extracellular GPX and gastrointestinal GPX.

Extracellular GPX was recognised as an enzyme distinct from cGPX in 1986 (Takahashi and Cohen, 1986). EGPX is also referred to as plasma GPX, and it consists of four identical 23 kDa subunits, each of which contains one selenocysteine. The function of EGPX is currently unknown, however it is known to be secreted by the kidneys, heart and lung.

Gastrointestinal GPX is found in the liver and gastrointestinal tract in humans. It is thought to decrease the absorption of hydroperoxides (Chu *et al*, 1993; Wingler *et al*, 1999). During Se deficiency the mRNA and the protein levels for GI-GPX remain stable, therefore Se is preferentially channelled to this selenoprotein (Wingler *et al*, 1999).

Skin GPX.

Recently a novel form of GPX has been found in the skin (Frank *et al*, 1997), this protein is upregulated by keratinocyte growth factor and is thought to be produced by fibroblasts and keratinocytes. It is thought that the protein is involved in wound repair, however this protein is not an selenoenzyme (Munz *et al*, 1997). The gene for this novel form of GPX has only slight identity to other GPX genes.

1.11.9 Thioredoxin Reductase.

Thioredoxin reductase is a flavin adenine dinucleotide-containing enzyme found in all organisms which, in conjunction with it's substrate thioredoxin, forms a redox system which has multiple functions including detoxification reactions (Holmgren and Bjornstedt, 1995; Tamura and Stadtman, 1996; Gladyshev *et al*, 1996). Thioredoxin reductase is a member of a larger family of pyridine nucleotide-disulphide oxidoreductases, which include lipoamide dehydrogenase, glutathione reductase (GR) and mercuric ion reductase (Zhong *et al*, 1998). The molecular mass of TR is 58 kDa and it has a dimeric structure, with each subunit containing a selenocysteine residue near the carboxyl terminus. (Gladyshev *et al*, 1996). Thioredoxin reductase reduces oxidised thioredoxin (a small multifunctional and ubiquitous protein) to reduced thioredoxin using NADPH as a cofactor. Protein disulphide

isomerase is also a substrate for TR (Lundstrom and Holmgren, 1990). The selenocysteine residue in the protein is essential for the reduction of thioredoxin (Gasdaska *et al*, 1996; Marcocci *et al*, 1997). Furthermore TR and thioredoxin have been shown to have growth regulating effects on cells (Berggren *et al*, 1996; Gallegos *et al*, 1996; Gasdaska *et al*, 1999). Thioredoxin and TR catalyse the transport of electrons to ribonucleotide reductase and other reductive enzymes. Ribonucleotide reductase is involved in the direct reduction of ribonucleotides to deoxyribobonucleotides, this is the rate-limiting step in DNA synthesis (Thelander and Reichard, 1979). Thioredoxin reductase can also detoxify lipid hydroperoxides and hydrogen peroxide (Bjornstedt *et al*, 1995a) (Fig 1.15). Thioredoxin reductase appears to have greater capacity to detoxify H_2O_2 and lipid peroxides than GPX. This suggests that one important function of TR is to protect cells from the oxidative damage of H_2O_2 and lipid hydroperoxides.

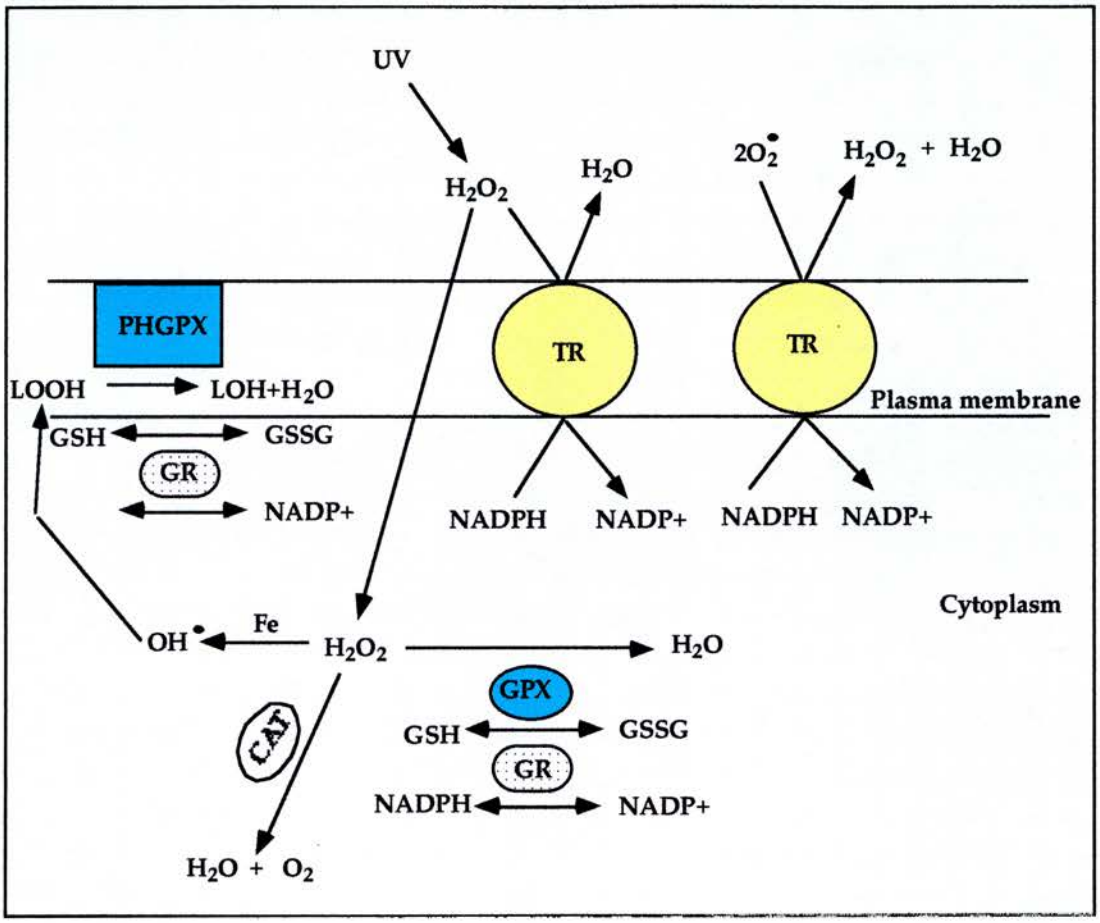
A number of TR enzymes have been sequenced from human placenta (Gasdaska *et al*, 1995), Jurkat cells (Gladyshev *et al*, 1996) and lung adenocarcinoma cells (Tamura and Stadtman, 1996). Recently it has been reported that three isoforms of TR exists. Two isoforms from rat liver have recently been cloned and sequenced.

As for other selenoproteins Se status can modify TR expression and activity (Berggren *et al*, 1997; Gallegos *et al*, 1997). A 3'-untranslated region, stem-loop sequence has been identified that may function as a putative SECIS (Brigelius-Flohe *et al*, 1994; Powis *et al*, 1997). Selenium deficiency can lead to a decrease in the levels of mRNA for TR and to incorporation of cysteine instead of selenocysteine into the protein (Gallegos *et al*, 1997). During Se deficiency in rats TR activity in the liver was found to decrease, whilst TR levels in the brain remain unchanged (Hill *et al*, 1997). Also *in vivo*, TR activity in rat liver, lung and kidney undergoes transitory increased expression when these animals are fed a high Se diet (Berggern *et al*, 1999). Changes in Se status, therefore result in altered expression of TR enzyme, which in turn may alter the growth characteristics or change the detoxifying capacity of the cell, leaving cells exposed to oxidative damage.

Thioredoxin reductase may also have effects on other antioxidant systems, it can induce manganese superoxide dismutase (Das *et al*, 1997) and regenerate

ascorbate from dehydrascorbate (May *et al*, 1997). The regenerated ascorbate can itself regenerate α-tocopherol from α-tocopherol semiquinone (Tamura *et al*, 1995), TR can also act as an electron donor to plasma GPX for the reduction of hydroperoxides (Bjornstedt *et al*, 1994).

Figure 1.15: Thioredoxin reductase and glutathione peroxidase activity in the skin.



High levels of selenite and selenodiglutathione are efficient oxidants of thioredoxin and can inhibit the function of the thioredoxin system (Bjornstedt *et al*, 1992; Kumar *et al*, 1992; Bjornstedt *et al*, 1995b). The inhibition of the thioredoxin system then leads to an inhibition of its many functions, such as the inhibition of ribonucleotide reductase and general protein disulphide reductase activity. These effects of the thioredoxin system may help to explain the growth inhibitory functions of high levels of some Se compounds (Bjornstedt *et al*, 1992; Ganther, 1999).

Extensive work has been carried out on the importance of the thioredoxin/TR system within the epidermis. Thioredoxin reductase is present on the plasma membrane of keratinocytes and melanocytes and it can protect the epidermis from the damaging effects of reactive oxygen species (Schallreuter and Wood, 1986; Schallreuter *et al*, 1986a). There is a link between reduction of free radicals by TR and melanin biosynthesis by melanocytes (Schallreuter and Wood, 1986). Schallreuter and Wood reported a direct correlation between TR activity and skin pigmentation. Thioredoxin reductase activity in patients with skin type VI (highly pigmented) is five fold higher than that found in patients with skin type I (fair skin) (Schallreuter *et al*, 1987). Thioredoxin reductase and thioredoxin levels increase in the skin following exposure to oxidative stress (Schallreuter *et al*, 1994). Furthermore TR can reduce superoxide to water and H_2O_2 (Schallreuter and Wood, 1986). Subsequently TR can reduce H_2O_2 to water (Schallreuter and Wood, 1989).

1.12 Selenium and the immune system.

Selenium can augment the activity of the immune system (reviewed in Spallholz *et al*; 1990; McKenzie *et al*, 1998). Mice maintained on Se supplemented diets, show an increased capacity for T cells to proliferate in response to stimulation with antigen or mitogen and to differentiate into cytotoxic effector cells (Meeker *et al*, 1985; Koller *et al*, 1986; Kiremidjian-Schumacher *et al*, 1992; Roy *et al*, 1992). The effects of Se on murine T cells have been reproduced in humans, with an increase in the levels of IL-2 receptor protein and natural killer cell function also detected (Kiremidjian-Schumacher and Roy, 1998). Human patients given 200 μg sodium selenite for 8 weeks, express more IL-2 receptor on the surface of their T cells than the unsupplemented control group (Roy *et al*, 1994). During Se deficiency antibody titres in mice decrease (Spallholz *et al*, 1973, 1975). This was also found to be the case in a more recent study, were Se-deficient rats where found to have lower antibody titres and a decreased delayed type hypersensitivity reaction (Kukreja and Khan, 1998).

1.13 Selenoproteins and the skin.

There is much evidence to suggest that Se has an important role in protecting skin from the harmful effects of UVB. In mice Se supplementation can substantially decrease the level of skin damage, tumour formation and overall mortality following UVB exposure (Overvad *et al*, 1985; Pence *et al*, 1994; Stewart *et al*, 1996). In man subnormal Se status is associated with up to a 4-fold increased risk of developing skin cancer (Reinhold *et al*, 1989; Clark *et al*, 1984; Deffaunt *et al*, 1994). Topical Se application, as selenomethionine, has been shown to protect human subjects and mice from acute skin damage and decrease sunburn cell formation following UVB exposure (Thorling *et al*, 1983; Burke *et al*, 1992a and b; LaRuche and Cesarini, 1991). In Se deficiency there is an increase in UVA-induced lipid peroxidation in cultured fibroblasts (Moysan *et al*, 1995). In keratinocyte cultures, selenite decreases UV-induced oxidative damage to DNA when added to the growth medium (Stewart *et al*, 1996). Leccia *et al*, have shown that incubation with selenite increases GPX activity and decreases UVA-induced lipid peroxidation in human skin fibroblasts (Leccia *et al*, 1993; Emonet-Piccardi *et al*, 1998). Furthermore, dietary supplementation in mice with sodium selenite, has been reported to increase GPX levels and decrease the incidence of chemically induced skin carcinogenesis (Perchellet *et al*, 1987). Topically applied thermal water from natural spas, containing a high level of Se decreases the level of UVB-induced lipid peroxidation and skin carcinogenesis in hairless mice (Overvad *et al*, 1985). Moreover, Se supplementation in patients diminish the levels of lipid peroxidation induced by exposure to UVB (Pietschmann *et al*, 1992). These studies will be discussed in more detail in the relevant result chapters of this thesis (reviewed in McKenzie, 2000).

1.14 Aims.

In this thesis the effect of Se on UVB-induced damage to the skin will be investigated. There is a great deal of evidence to suggest that Se is crucial in protecting the skin from the harmful effect of UVB. Se supplementation has been shown to prevent UVB-induced tumourogenesis and immunosuppression in the skin. Therefore the aim of this thesis was to investigate how Se affects the skin. To achieve this aim the effect of Se on

many different UVB-induced types of damage in the skin have been studied, including:

- Necrotic cell death
- The formation of lipid peroxides
- Apoptotic cell death
- p53 protein expression
- DNA damage
- Cytokine expression
- LC migration

Selenoprotein distribution and expression within the skin was also investigated.

Chapter 2

General Materials and Methods

2.1 Materials.

Cell Culture Reagents.

Dulbecco's modified Eagle's media (DMEM), penicillin-streptomycin (pen-strep), glutamine, dispase, trypsin, sodium pyruvate, keratinocyte serum-free media (K-SFM), Earle's balanced salt solution (EBSS), lymphocyte separation media and fungizone were purchased from Gibco Life Technologies BRL, Paisley, Renfrewshire, UK. The melanocyte culture media was purchased from Sigma-Aldrich, Poole, Dorset, UK. Tissue culture plastics were supplied by Corning, High Wycombe, UK.

Radioisotopes.

The [^{75}Se]-selenite (33 MBq/mg) was purchased from the University of Missouri, Columbia, USA. Adenosine triphosphate (ATP) [gamma ^{32}P] was purchased from ICN Biomedicals, Basingstoke, UK.

Immunochemicals.

Antibodies to rat TR and rat PHGPX were raised in rabbits using proteins purified as described previously (Holmgren and Bjornstedt, 1995; Roveri *et al*, 1994). The antibody for human p53 (DO7) was purchased from TCS Biologicals Ltd, Buckingham, UK). The avidin-biotin complex (ABC)-Horseradish peroxidase (HRP) was obtained from DAKO, High Wycombe, UK. Enzyme-linked immunosorbent assays (ELISA) kits were purchased from Genzyme, Cambridge, USA. Monoclonal rat anti-mouse IL-10 antibody was purchased from Harlan Sera-Lab, Belton, UK. Biotinylated anti-rat IgG raised in rabbit was obtained from Vector, Burlingham, USA. Enhanced chemiluminescence reagents (ECL) were obtained from Amersham Life Science, Chalfont, UK. Anti-mouse IgG-HRP conjugate was

purchased from Autogen Bioclear, Wiltshire, UK. Slide mounting solution DePeX, was from Merck, Poole, UK.

General materials.

Aprotinin and bovine serum albumin (BSA), were purchased from Boehringer Mannheim, Germany. All primers, the oligo $dt_{(18)}$, RNA guard, nitrocellulose and dinucleotide triphosphates (dNTPs) were purchased from Pharmacia Biotech, St Albans, UK. Thermus icelandicus red hot DNA polymerase was purchased from Advanced Biotechnologies, Epsom, UK. AGFA film developer and fixer were from HA West (X-ray) Ltd, Edinburgh, UK. The T4 polynucleotide kinase (PNK) was obtained from New England Biolabs, Hitchin, UK. Agarose, guanidine isothiocyanate, HEPES, dithiolthreitol (DTT) and Molony murine leukaemia virus reverse transcriptase (MMLV) were purchased from Gibco Life Technologies BRL, Paisley, Renfrewshire, UK. All other chemicals and reagents were purchased from Sigma-Aldrich, Poole, Dorset, UK.

2.2 General methods.

Specific methods will be covered in the individual results chapters.

2.2.1 Culture conditions for cell lines.

Cultures of the spontaneously transformed human keratinocyte line HaCaT (Boukamp *et al*, 1988) (gift from Professor N Fusenig, Differenzierung und Carcinogenese, Deutsches Krebsforschungszentrum), were grown in DMEM, supplemented with 5% foetal calf serum (FCS), 100 units/ml penicillin -100 µg/ml streptomycin in a humidified 5% carbon dioxide (CO_2) atmosphere at 37°C. Cultures of the spontaneously transformed mouse keratinocyte cell line PAM 212 (Yuspa *et al*, 1980), the human epithelial carcinoma cell line A431 (Giard *et al*, 1973), human epithelial cell line HeLa (derived from a carcinoma of the cervix) (Scherer *et al*, 1953) and the human epithelial cell line SiHa (derived from a squamous cell carcinoma of the uterus) (Friedl *et al*, 1970) were also grown in the same conditions as HaCaT cells.

2.2.2 Primary fibroblasts.

Primary fibroblasts were cultured from forearm biopsies (Sly and Grubb, 1979). The dermis was isolated and dissected into 1 mm^2 sections. The small sections were placed underneath sterilised glass coverslips in 10 cm^2 petri dishes. The petri dishes were flooded with DMEM containing 10% FCS, 2.5 μg/ml fungizone and Pen-Strep (as section 2.2.1) and placed in a humidified 5% CO_2 atmosphere at 37°C. The dishes were left undisturbed for 2 weeks, after which time the media in the dishes was changed every 2 days. After 4-6 weeks the fibroblasts began to migrate out from beneath the coverslips, the coverslips were then removed and the fibroblasts cultured until confluent. Fibroblasts will grow indefinitely, however in this project they were used between passages 3-6 only.

2.2.3 Primary keratinocytes.

Primary human keratinocytes were cultured from neonatal foreskins, obtained from the Royal Hospital for Sick Children, Edinburgh (Kondo *et al*, 1993). The foreskin samples were incubated overnight in 0.1% dispase/DMEM solution. The epidermis was removed and dispersed in trypsin (0.5 g/L) and 0.68 mM tetrasodium ethylenediaminetetracetic acid (EDTA) for 15 minutes. Cells were then plated out on fibronectin-coated plates (5 $\mu g/cm^2$) and grown in K-SFM containing Pen-Strep (as in section 2.2.1). After 2 passages the cultures were composed predominantly (>95%) of keratinocytes. The cells were used at passage 3 or 4.

2.2.4 Primary Melanocytes.

Primary melanocytes were isolated from neonatal foreskins also. The foreskin samples were treated as for keratinocyte isolation, however following treatment with trypsin to disperse the epidermis, the cells were plated into melanocyte media (Sigma-Aldrich, Poole, Dorset, UK), containing pen-strep (as in section 2.2.1). The cultures were incubated overnight and then trypsinised for 1 minute only, this was sufficient time to dislodge the melanocytes, which were then plated into fresh flasks and maintained in melanocyte media. The melanocytes were also used at passage 3 or 4.

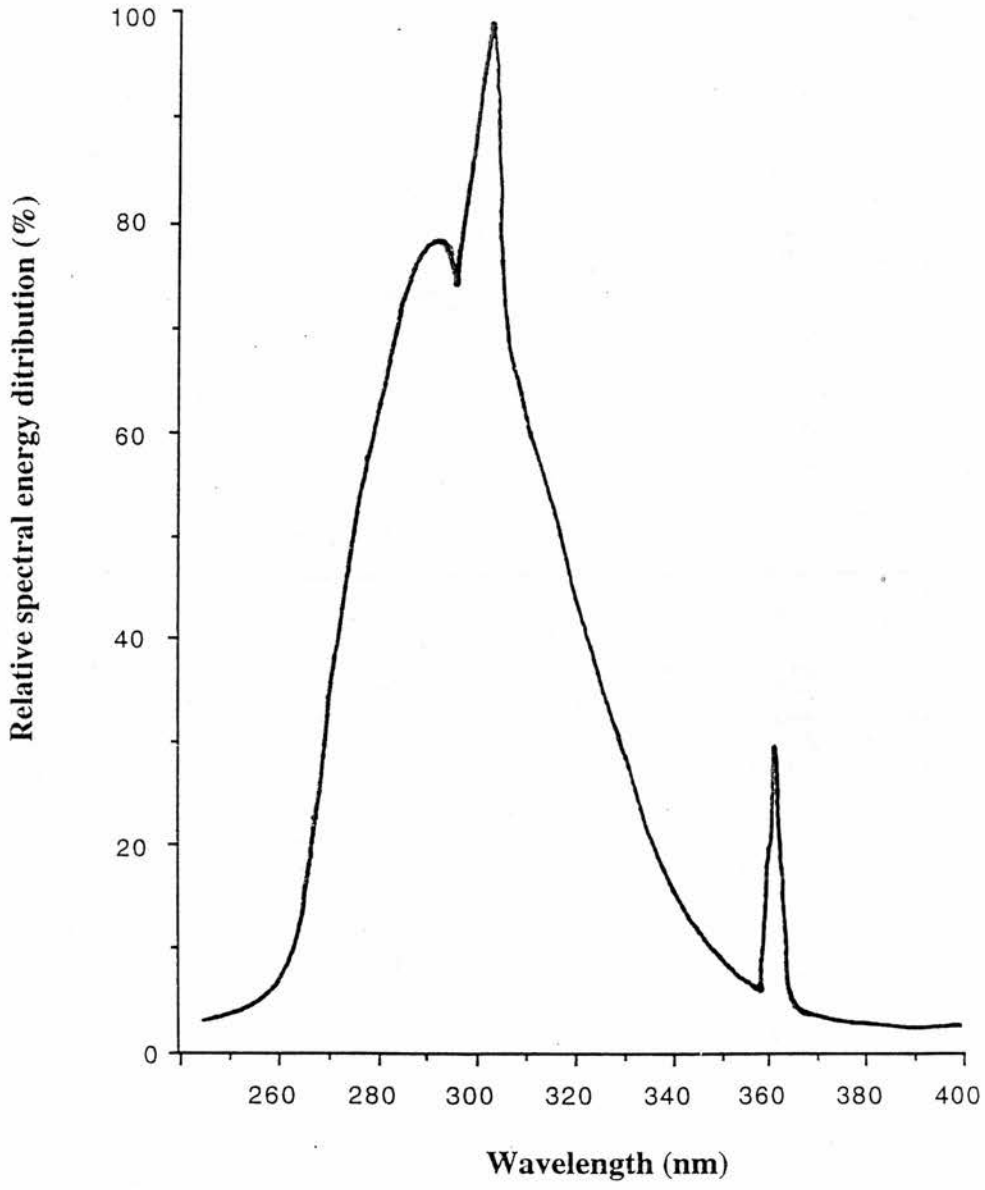
2.2.5 Measurement of Se content of individual cell culture media, skin samples and plasma.

The Se contents of each culture media were determined by acid digestion followed by fluorimetric analysis (Olsen *et al*, 1975; Boyne and Arthur, 1986) this was carried out by Dr J Arthur, Rowett Research Institute, Aberdeen, UK. The samples were digested overnight in 2 ml of concentrated nitric acid. The samples were then heated slowly to boiling point and held at boiling point for 5 minutes. Concentrated perchloric acid (2 ml) was then added dropwise to the samples and the samples were boiled again for 30 minutes. Then 2 ml of 10% volume/volume (v/v) hydrochloric acid (HCl) was added to convert Se as selenate to selenite. The samples were then cooled and 5 ml hydroxylamine/EDTA solution (25 g hydroxylamine and 9.24 g EDTA/L distilled water) was added to each sample, followed by 5 drops of cresol red (50 mg cresol red dissolved in distilled water (250 ml) containing 1 ml of 40% (v/v) ammonia). Ammonia solution 40% (v/v) was added to each sample until the sample colour changed from red to green (approximately 3 ml), then 10% (v/v) HCl was added until the colour of samples changed from green to orange. This brought the pH of the samples to 1.5-2.5 which allows the formation of the diaminonaphthaline-Se complexes. The samples were then diluted to 50 ml with distilled water and 5 ml of diaminonaphthaline solution was added, the samples were then incubated for 30 minutes at 50°C. The samples were then cooled and 6 ml cyclohexane added, the samples were then shaken for 20 seconds to extract the daminonaphthaline-Se complexes. The samples were left to stand to allow the diaminonaphthaline layer to separate. The top layer containing the Se-complexes was then removed and the fluorescence measured. Dilution's of selenous acid were used to form a standard curve.

2.2.6 Ultraviolet irradiation.

Before all exposures to ultraviolet radiation cells were grown to 70% confluence and the media was removed, set aside and the monolayer was covered with phosphate buffered saline (PBS). With the exception of the experiments described in Chapter 5, cells were irradiated with broadband UVB from a bank of two TL-20W/12 lamps (Philips, Croydon, UK) with an

Figure 2.1: Emission Spectrum for TL-20W/ 12 UVB Lamps.



The output of the lamps was determined by Dr Neil Gibbs (Photobiology Unit, Ninewells Hospital, Dundee) using a spectroradiometer (model 742, Optronic Laboratories) across the spectral range 250-400 nm. The tube target distance was 16 cm.

output range of 270-350 nm (peak 308 nm) (Fig 2.1). The irradiance was 80 mW/cm^2 at a distance of 30 cm (Kondo *et al*, 1993).

The experiments in Chapter 5 where carried out in the MRC Cell Mutation Unit, Sussex University, Brighton and the lamps used have a similar spectral output to the TL-20W/12 lamps, however they have emit less UVC than the TL-20W/12 lamps. They emit broadband UVB, from a bank of four FS-20 Westinghouse lamps (Clingen *et al*, 1995). For some experiments the lamps were shielded with plastic petri dishes, to decrease the output to allow very low doses of UV radiation to be given accurately (Fig 2.2).

During all experiments an IL-1400A radiometer, equipped with a SEL240/UVB 1/TD UVB detector with a spectral sensitivity in the range of 280-320 nm (International light Inc, Montreal, Canada) was used to quantify the UVB joules/ $metre^2$ (J/m^2) output that the cells or mice were exposed to.

2.2.7 Solutions of Se compounds.

Stock solutions of sodium selenite and seleno-DL-methionine were made in PBS, at concentrations 100-1000 fold of the working concentrations. The solutions were stored at -20°C after being filter sterilised.

2.3 Methods for measuring cell survival and lipid peroxidation.

2.3.1 Trypan blue exclusion assay.

Following exposure of the cells to the toxic insult, the original media was replaced. Cell viability was determined by cell counts 48 hours later. The cells were trypsinised and resuspended in 50 µl PBS and 50 µl trypan blue, 0.4% weight/volume (w/v) then the cells were counted on a haemocytometer. At least 100 cells were counted for each point, n=3.

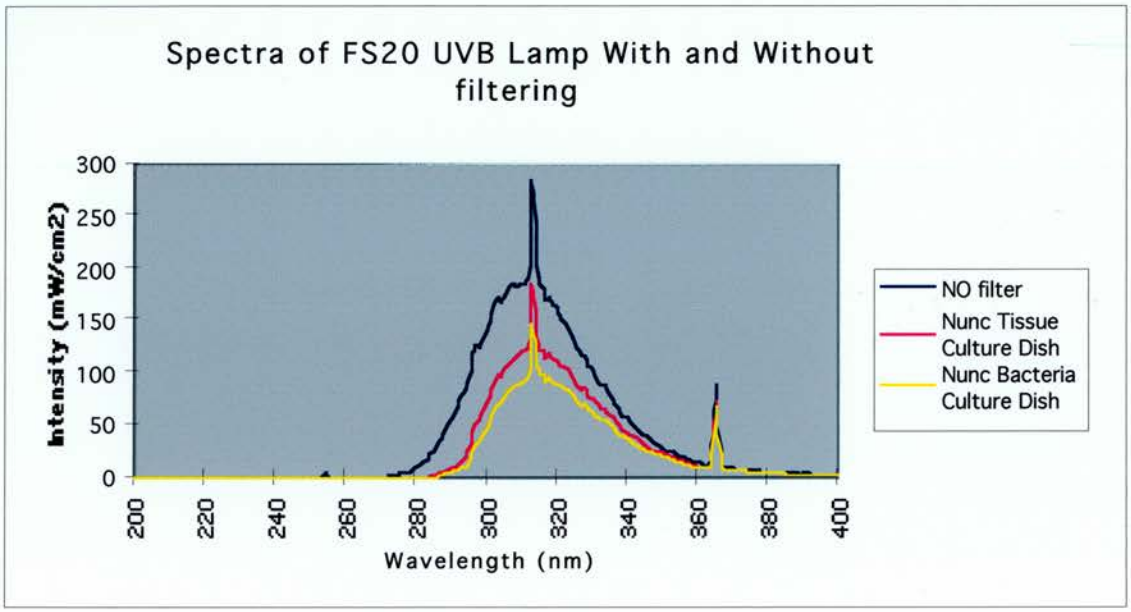
2.3.2 Effect of Se supplementation on cell viability and growth.

Growth stimulation by Se was assessed by counting cells using a Coulter counter, model: DN (Coulter electronics Ltd, Harpenden, UK). Cell viability was monitored using the trypan blue exclusion method (section 2.3.1).

Figure 2.2: Emission Spectra for FS20 UVB Lamps.

Readings were taken using a Bentham Spectroradiometer, double monochromater with integration sphere.

Source - 4 Westinghouse FS20 lamps 9 cm from light meter



	FS20 No Filter	Tissue culture	Bacterial culture
% UVA	47.79	57.35	60.64
% UVB	51.67	42.65	39.36
% UVC	0.54	0.00	0.01
Total Intensity	8056.82	5024.99	3989.25

Tissue culture dishes allowed 62.37% of the total lamp output through.
Bacterial culture dishes allowed 49.51% of the total lamp output through.

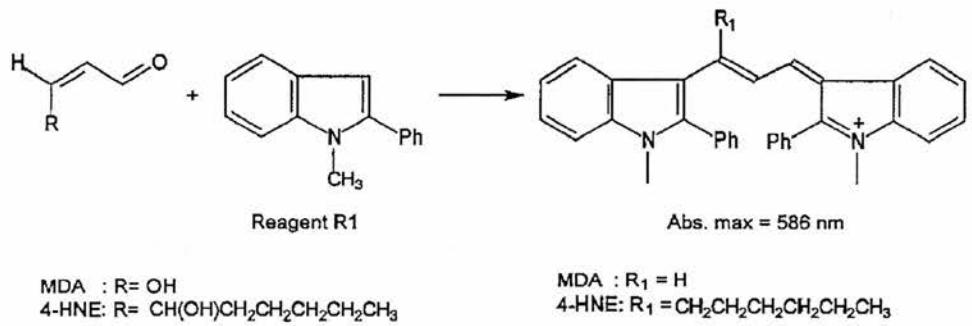
2.3.3 Colony-formation assay.

Keratinocytes and fibroblasts in logarithmic growth phase were counted on a haemocytometer. Cells were plated at $1x10^3$ cells in 10 cm tissue culture petri dishes. Cells were allowed to attach for 24 hours before being exposed to the damaging agent. If the cells were exposed to UVB, the media was removed and replaced with PBS during the irradiation. The original media was then replaced for 48 hours, the media was then replaced again and the cells and left for 7-14 days until colonies had formed on the control plates. The media was then aspirated, the cells washed in PBS, and stained with 1% (w/v) crystal violet reconstituted in 20% (v/v) ethanol for one minute. The plates were then washed extensively in water and the number of colonies with >25 cells counted on a See-scan image analysis system (Seescan, Cambridge, UK). Triplicate dishes were counted for each point.

2.3.4 Lipid peroxidation assay.

The cells were harvested into PBS, then lysed by repetitive freeze thawing, prior to level of lipid peroxidation being measured using a kit from Calbiochem-Novabiochem Ltd, Nottingham, UK. Samples were analysed according to the manufacturer's instructions. The principal of the kit is that the malondialdehyde (MDA) in the samples, reacts with reagent R1 in the kit to form a stable chromophore with maximal absorbance at 586 nm (Philips spectrophotometer model PU8625) (Fig 2.3). A standard curve was constructed using dilution's of pure MDA.

Figure 2.3: Lipid Peroxidation Assay Kit Reaction.



Two molecules of chromogenic reagent R1, react with one molecule of MDA or 4-hydroxyalkenal, at 45°C yielding a stable chromophore with maximal absorbance at 586 nm.

2.4 Methods used to study apoptosis and p53 expression.

2.4.1 Morphological detection of apoptotic cells using acridine orange.

Cells were cultured in 6 well dishes, then the monolayers and culture media supernatants were harvested separately and centrifuged at 800 g for 10 minutes. Cells were resuspended in 20 µl PBS and initial cell counts carried out using a haemocytometer. Apoptotic cells were identified by staining with an equal volume of the DNA binding fluorophore, acridine orange (5 µg/ml in physiological saline) and visualised using fluorescent microscopy (Leitz, Otholux II), with a FITC-3 fluorescent filter (excitation filer, Wratten 47B and barrier filter, Wratten 12, Kodak). The number of apoptotic cells in samples were counted over 20 microscope fields and calculated as the percentage of the total number of cells present in both the supernatants and in the monolayers, n=3.

2.4.2 Electron microscopy.

The cells were fixed in 3% glutaraldehyde diluted in 200 mM sodium cacodylate buffer (pH 7.4) for 30 minutes, before being washed in 200 mM sodium cacodylate buffer (pH 7.4) for 15 minutes (between each wash and all subsequent treatments the cells were centrifuged at 2000 rpm for 5 minutes). The cells were then fixed in 1% osmium tetroxide prepared in 200 mM sodium cacodylate buffer (pH 7.4) for 30 minutes, then washed in 200 mM sodium cacodylate as previously. The cells were dehydrated in 10% ethanol for 5 minutes, this was repeated 3 times. The cells were further dehydrated in absolute ethanol for 20 minutes, again this was repeated three times. Finally the cells were treated with epoxy propane twice for 10 minutes, before being embedded in araldite.

Thin sections (1 µm) were then cut from the embedded cells and examined using an electron transmission microscope (Philips, model: CM12, Croydon, UK).

2.4.3 Detection of internucleosomal cleavage during apoptosis.

Cells ($1x10^6$) were suspended in 20 µl of lysis buffer (10 mM EDTA, 50 mM Tris-HCl pH 8.0, 0.5% (w/v) sodium lauryl sarcosinate and 0.5 µg/ml proteinase K) and incubated at 50°C for 1 hour. After which time 10 µl of 0.5 mg/ml RNase A was added and the cells incubated for a further hour at 50°C. Cell samples were then incubated with 10 µl of 10 mM EDTA (pH 8.0) containing 1% low melting point agarose for 5 minutes at 70°C, prior to loading on a 2% agarose gel containing 35 µg/ml ethidium bromide. The samples were allowed to solidify for 5 minutes prior to electrophoresis with 90 mM Tris-phosphate, 2 mM EDTA as buffer, at 40 Volts for 3-4 hours. The ethidium-stained DNA laddering was visualised using a UV transilluminator.

2.4.4 Western blot analysis for p53.

2.4.4.1 Preparation of cell lysates.

Primary keratinocytes and A431 cells were lysed using triple lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 0.02% sodium azide, 0.1% sodium dodecyl sulphate (SDS), 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml Aprotinin, 0.5% (w/v) sodium deoxycholate, 1% (v/v) IGEPAL) and then centrifuged for 5 minutes at 10000 g to pellet any cellular debris. Resulting supernatants were aspirated and stored at -20°C. Protein levels were measured using the microlowry protein assay (Lowry *et al*, 1951).

2.4.4.2 Gel electrophoresis and western blotting.

Equal amounts of protein (10 µg) for each sample were run on 10% SDS-polyacrylamide gels (SDS-PAGE) using 1x loading buffer (2% SDS, 100 mM DTT, 60 mM Tris-HCl pH 6.8, 0.01% bromophenol blue), the samples were boiled for 5 minutes prior to being loaded onto the gel. Electrophoresis was carried out at 100 Volts, using 25 mM Tris- 250 mM glycine with 0.1% (w/v) SDS as the electrophoresis buffer. The proteins were then transferred onto nitrocellulose (Biorad, Herts, UK) at 100 Volts for 1 hour in the presence of transfer buffer (25 mM Tris, 190 mM glycine and 20% methanol). For p53

detection, the nitrocellulose was blocked using 4% non-fat Marvel (powdered milk) dissolved in Tris buffered saline with Tween (TBS-T) (50 mM Tris-HCl, 150 mM NaCl, pH 7.6, 0.2% (v/v) Tween-20) for 1 hour at room temperature. This was followed by overnight incubation at 4°C with mouse monoclonal IgG for human p53 primary antibody (DO7) diluted 1:1000 in 4% milk/TBS-T. The mouse antibody recognises both wild type and mutant forms of human p53. The nitrocellulose was subsequently washed 4 times for 10 minutes in TBS-T, before incubation for 2 hours at room temperature with a 1:2000 dilution of an anti-mouse IgG-HRP conjugated antibody in 4% milk/TBS-T. The nitrocellulose was then washed 4 times for 10 minutes in TBS-T, with one final wash in TBS. Blots were then visualised using ECL and Kodak X-OMAT-XAR autoradiography film. Films were then quantitated using a Seescan system image analyser (Seescan, Cambridge, UK) and integrated OD units were obtained using gel analysis software v1.0 1D (Seescan).

2.4.5 Immunostaining for p53.

Cells were grown on sterile glass coverslips in 6 well dishes until 70% confluent, then treated with either UVB alone or Se and UVB according to individual methods described in Chapter 4. The cells were then fixed in 4% paraformaldehyde in PBS for 10 minutes. Following fixation the cells were washed twice in PBS and treated with 100% ethanol followed by, treatment with methanol containing 1% H_2O_2 for 10 minutes, to block endogenous peroxidase. The coverslips were then rinsed in water and blocked using 20% rabbit serum for 10 minutes. The mouse monoclonal primary antibody for human p53 (DO7) was incubated with the coverslips at a dilution of 1:200 overnight at 4°C. The cells were then rinsed in PBS twice for 5 minutes and exposed to a 1:400 dilution of a HRP-conjugated rabbit anti-mouse polyclonal secondary antibody for 30 minutes, at room temperature. The coverslips were rinsed in PBS twice before exposure to ABC-HRP for 30 minutes (carried out as per kit instructions, DAKO, High Wycombe, UK). Cells were rinsed in PBS twice for 5 minutes prior to exposure to diaminobenzidine for 5 minutes (as per kit instructions, Sigma-Aldrich, Poole, Dorset, UK). Finally the cells were dehydrated by passing the coverslips through graded alcohol's and finally into xylene, prior to being

mounted using the synthetic resin DePeX (Merck, Poole, UK). Positive staining appeared brown.

2.5 Comet Assays.

2.5.1 Preparation of cells and irradiation.

The comet assay was performed as outlined previously (Singh *et al*, 1988; Green *et al*, 1996). Normal agarose (0.6% in PBS) (85 µl) was layered onto frosted microscope slides using 22 x 22 mm coverslips. Cells were then trypsinised (1% trypsin (w/v) and 0.4% EDTA for 3-5 minutes at 37°C) and resuspended at a density of $4x10^5$ cells/ml with 0.6% low melting point agarose prepared in PBS. The cell suspension was added as a second layer to each slide using 50 µl. Slides were maintained and irradiated on ice (exposure levels are stated in chapter 5 for each experiment). The slides were then processed using one of the comet assays as detailed below.

2.5.2 Excision repair.

After irradiation, if excision repair was being studied, 100 µM cytosine arabino furanoside and 10 mM hydroxyurea in PBS were added to the slides and incubated at 37°C for 1 hour. The slides, were immersed in lysis solution (2.5 M sodium chloride, 200 mM sodium hydroxide, 100 mM EDTA, 10 mM Tris, 10% (v/v) dimethyl sulfoxide and 1% (v/v) Triton X-100, pH 10) at 4°C for at least 1 hour. Slides were then placed in alkali electrophoresis buffer (300 mM sodium hydroxide, 1 mM EDTA) and left for 40 minutes to allow the DNA to unwind at 10°C. The slides were then electrophoresed at 20 Volts for 24 minutes at 10°C. Following electrophoresis, slides were washed with neutralising Tris buffer (400 mM, Tris-HCl, pH 7.5) and stained with 30 µl of ethidium bromide solution (20 µg/ml). Comet length was determined using the Casys system (Synoptics, Cambridge, UK).

2.5.3 The T4 endonuclease comet assay.

The cells were prepared as previously described in section 2.5.1 and irradiated, however following irradiation the slides were immersed in lysis buffer immediately and incubated as in section 2.5.2. Following lysis the

slides were neutralised using T4 endonuclease buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA and 75 mM sodium chloride). For treatment with DNA repair enzymes, cells which were embedded in agarose, were overlaid with 50 µl of T4 endonuclease V (5-20 µg/ml) (kindly supplied by Applied Genetics, Freeport, NY). Cover slips were added and the slides incubated for 1 hour at 37°C in a humidified atmosphere. Time course and concentration dependence studies were performed with all batches of enzyme to ensure that the amount of enzyme activity was saturating. Control slides were incubated as above with 50 µl of T4 endonuclease buffer. Following treatment with enzyme the slides were placed in alkaline electrophoresis buffer and immediately electrophoresed, neutralised and visualised as in section 2.5.2.

2.5.4 Foramidopyrimidine-DNA Glycosylase (FaPy-glycosylase) comet assay.

This assay was carried out almost identically to the T4 endonuclease assay; except that the T4 endonuclease buffer, was replaced with FaPy glycosylase buffer (40 mM HEPES pH 8.0, 100 mM potassium chloride, 0.5 mM EDTA and 200 µg/ml BSA). Also the FaPy enzyme (kindly supplied by A. Collins, Aberdeen, UK) was used instead of the T4 endonuclease V (Collins *et al*, 1993).

2.6 Cytokine analysis.

2.6.1 RT-PCR.

2.6.1.1 Cell lysis.

Following exposure to UVB the cells were incubated for 6, 12 or 24 hours prior to being washed in PBS and lysed in ice-cold Reagent D (4 M guanidinium isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sodium lauryl sarkosinate, 0.1 M 2-mercaptoethanol) and stored at -70°C.

2.6.1.2 RNA extraction.

Total ribonucleic acid (RNA) was extracted by using a phenol-chloroform procedure (Chomczynski and Sacchi, 1987). The samples were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:25:1, v/v) and precipitated with one tenth the volume of 2 M sodium acetate for 1 hour at -70°C in isopropanol. The RNA was subsequently washed in 70% ethanol, dried and dissolved in 40 µl distilled water and quantitated by its absorbance at 260 nm (Philips spectrophotometer model PU8625).

2.6.1.3 Reverse transcription.

To generate the complementary DNA (cDNA) 2 µg of RNA was denatured at 65°C for 2 minutes. Then 1x reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 75 mM potassium chloride, 3 mM magnesium chloride [$MgCl_2$]), 0.5 mM of each dNTP, 30 units of human placental RNase inhibitor, 200 units of MMLV reverse transcriptase, 1 µg oligo $dT_{(18)}$, and 10 mM DTT and water were added to give a final reaction volume of 20 µl. The reaction mix was then incubated at 37°C for 1 hour and then the reaction was terminated by heating to 95°C for 5 minutes.

2.6.1.4 Primer labelling.

Each primer was end-labelled with γ-[^{32}P]-dATP in a 30 µl reaction mix consisting of 5 µM primer, 5 mM DTT, 100 units T4 PNK, 1x PNK buffer (70 mM Tris-HCl, 10 mM $MgCl_2$) and 5 µl of 0.08 mCi/ml γ-[^{32}P]-dATP at 37°C for 1 hour. The labelling reaction was terminated by heating to 95°C for 5 minutes.

2.6.1.5 PCR procedure.

The polymerase chain reaction (PCR) was performed using 2 µl of the reverse transcriptase reaction in a PCR cocktail containing 0.5 units Red-hot DNA polymerase, 1x PCR buffer (20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl, 0.01% Tween), 0.08 mM of a mix of all four dNTPs, 0.375 µM of each primer and 1 µM tetramethylammonium chloride. Each primer set was optimised for $MgCl_2$ concentration (Listed in Table 2.1) and the volume for each PCR

reaction was made up to 10 µl with water. PCR reactions were overlayed with 20 µl of mineral oil to prevent evaporation and were amplified on a Techne PHC-3 or Gene-E dri block cycler (Techne, Cambridge, UK). The cycling times were 1 minute at 95°C (denaturation), 1 minute at 60°C (primer annealing) and 1 minute at 72°C (extension). The number of cycles for each primer, were calculated by determining the number of cycles which produced an exponential amount of product without saturating the reaction, this tended to be between 30-40 cycles. (Primer sequences, $MgCl_2$ requirements and product sizes are found in Table 2.1).

2.6.1.6 Quantitation of PCR products.

Radioactive PCR products were resolved on 12% SDS-PAGE gels (150 Volts) and identified by size, in comparison to 100 base pair ladder (Gibco Life Technologies BRL, Paisley, Renfrewshire, UK). The gels were dried and exposed using a molecular phosphoimager, model: GS-525 (Biorad, Hemel Hempstead, Herts, UK) and visualised by autoradiography using Kodak X-OMAT XAR-5 film. The house keeping genes G3pDH and β-actin were used when studying human and mouse cells respectively, to control for differences in the abundance of cDNA. The values for each test cytokine were normalised using the respective house-keeping gene value for each sample. All samples and controls were amplified within the same PCR run and subjected to SDS-PAGE and exposed on the phosphoimager at the same time.

2.6.1.7 Controls.

The RNA from EL-4 cells (mouse T cell line) stimulated with phorbol-12-myristate-13-acetate (PMA) (50 ng/ml) served as a positive control for the mouse cytokines. The RNA isolated from primary human T cells stimulated with PMA served as the positive controls for the experiments investigating human cytokine production. The primary human T cells were isolated from human buffy coat preparations obtained from the Blood Transfusion Service at the Royal Infirmary, Edinburgh. The white blood cells were isolated by layering the buffy coats onto lymphocyte separation media and centrifuging at 1500 rpm for 30 minutes. The white blood cells were then transferred to a fresh tube and washed three times in RPMI and resuspended in RPMI

containing 10% FCS. The T cells were subsequently stimulated for 24 hours with PMA (50 ng/ml) and phytohaemagglutinin (1 μg/ml). After stimulation the RNA was extracted as described previously. PCR reactions where distilled water replaced cDNA, were used to check for any contaminants in the PCR reagents.

Table 2.1: Cytokine primer pairs.

Human Cytokines	Primer Sequence Read 5'-3'	Product Size (bp)	$MgCl_2$ (mM)
TNF-α	5'= ATGAGCACTGAAAGCATGATCCGG 3'= GCAATGATCCCAAAGTAGACCTGCCC	695	2.39
IL-8	5'= ATGACTTCCAAGCTGGCCGTGGCT 3'= TCTCAGCCCTCTTCAAAAACTTCTC	289	2.5
G3pDH	5'= TGAAGGTCGGAGTCAACGGATTTGGT 3'= CATGTGGGCCATGAGGTCCACCAC	983	2.39
IL-1α	5'= ATGGCCAAAGTTCCAGACATGTTTG 3'= GGTTTTCCAGTATCTGAAAGTCAGT	816	2.39
IL-6	5'= ATCAACTCCTTCTCCACAAGCGC 3'= GAAGAGCCCTCAGGCTGGACTG	628	2.39
Mouse cytokines			
β-actin	5'= GTGGGCCGCTCTAGGCACCAA 3'= CTCTTTGATGTCACGCACGATTTC	540	2.39
IL-10	5'= ACCTGGTAGAAGTGATGCCCCAGGCA 3'= CTATGCAGTTGATGAAGATGTCAAA	237	2.5
TNF-α	5'= ATGAGCACAGAAAGCATGATCCGC 3'= CCAAAGTAGACCTGCCCGGACTC	692	2.5

Primer pairs spanned at least one exon to amplify cDNA and not genomic DNA.

2.6.2 Cytokine quantification of cell supernatants by ELISA.

Cells were grown until 70% confluent and treated with Se for 24 hours prior to exposure to UVB. The original media was then returned to the cells and

the cells were incubated for 6, 12 or 24 hours, after which time the cell supernatants were removed and spun down for 1 minute at 10000 g to pellet any dead cells that may be present. The supernatant was aliquoted and stored at -70°C until required. The samples were analysed in triplicate using Predicta ELISA kits (Genzyme Corporation, Cambridge, MA, USA) according to the manufacturers instructions.

2.6.3 Immunostaining for IL-10.

Immunostaining for IL-10 was carried out as described in Section 2.4.5. Immunostaining was performed using a rat monoclonal anti-mouse IL-10 antibody, diluted 1:100 before overnight incubation at 4°C. In these experiments, the secondary antibody used was a biotinylated anti-rat IgG raised in rabbits, used at a 1:100 dilution for 30 minutes at room temperature.

2.7 Mouse Experiments.

2.7.1 Composition of Se diets.

The mouse diets were provided by Dr. J. Arthur, Rowett Research Institute, Aberdeen, UK. The composition of the experimental mouse diet was as described below in Table 2.2 (Arthur *et al*, 1987):

The basal diet contained either no Se, 0.1 ppm or 2 ppm Se added as sodium selenite where appropriate.

Table 2.2 : Composition of basal experimental mouse diet.

Constituent	Composition (g/100 g of diet)
Sucrose	71.8
Amino acids*	18.0
Lard	3.5
Cod liver oil	1.5
Vitamins, minerals and trace elements†	5.2

* The amino acid mixture contained the following per 113.0 g of mix : L-alanine, 5.0 g, L-arginine hydrochloride, 6.0 g, L-asparagine, 4.0 g, L-aspartic acid, 5.0 g, L-cysteine, 2.0 g, glycine, 5.0 g, L-histidine, 3.0 g, L-isoleucine, 5.0 g, L-leucine, 7.5 g, L-lysine hydrochloride, 7.0 g, L-methionine, 4.0 g, L-monosodium glutamate, 30.0 g, L-phenylalanine, 5.0 g, L-proline, 4.0 g, L-serine, 5.0 g, L-threonine, 5.0 g, L-tryptophan, 1.5 g, L-tyrosine, 3.0 g and L-valine, 6.0 g.

† The Vitamin mix contained (g/50 g mix): thiamin, 0.5 g, pyridoxine, 0.5 g, riboflavin, 0.5 g, p-aminobenzoic acid, 0.5 g, nicotinic acid, 1.5 g, calcium-pantothenate, 1 g, folic acid, 0.25 g, biotin, 0.25 g, inositol, 20 g, and sucrose, 25 g. The trace elements mix contained (g/250 g mix): KIO_3, 0.84 g, NaF, 0.277 g, NH_4VO_3, 0.023 g, $NiCl_2{\cdot}6H_2O$, 0.23 g, $Cr(SO_4)_3K_2SO_4{\cdot}24H_2O$, 2.4 g, $SnCl_4{\cdot}5H_2O$, 0.029 g and sucrose, 246.71g and omitted sodium selenite where appropriate (Abdel-Rahim *et al*, 1986).

2.7.2 Source of mice.

The mouse strain used were C3H/HeN female mice obtained from the specific-pathogen-free animal breeding facility at the department of Medical Microbiology Transgenic Unit, University of Edinburgh. Mice were weaned at the start of the experiment and were immediately transferred onto one of the Se diets for 6 weeks, prior to experimental procedures being carried out.

2.7.3 ATPase staining of epidermal sheets for LCs.

Twenty four hours following irradiation the mice were sacrificed and the ears collected. The ears were divided in two and were floated dermal side down in 0.76% EDTA pH 7.2 at 37°C for 2 hours. The ears were then rinsed three times in saline and the epidermal sheets were removed. The epidermal sheets were fixed in 1.28% sodium cacodylate/4% formaldehyde/6.85% sucrose at 4°C for 1 hour before being rinsed three times in saline. The epidermal sheets were then stained with adenosine diphosphate (ADP)-lead (0.6 mg/ml ADP, 0.125% lead nitrate, diluted in 1.2% magnesium sulphate, 5% glucose and 40% Tris-Mal buffer) (3% Tris, 2.9% maleic acid and 1% sodium hydroxide) at 37°C for 70 minutes and then rinsed in Tris-Mal buffer three times before treatment with 2% ammonium polysulfide for 20 minutes

at room temperature. Finally the epidermal sheets were rinsed in water and mounted on slides in 50% glycerol (Juhlin and Shelley, 1977; Chaker *et al*, 1984). The number of LCs were counted in 10 fields/epidermal sheet.

2.7.4 Protein Determination.

Mouse tissue samples, cellular extracts for analysis of selenoprotein profiles and enzyme activities were sonicated and the protein levels determined using the Bradford dye-binding method (Bradford, 1976) adapted for use on a Cobras Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK). Bradford reagent was made by dissolving 0.01% comassie brilliant blue G-250 in 5% ethanol and 8.5% phosphoric acid, the solution was then filtered through a Whatman grade 1 filter paper.

Bradford reagent (265 µl) and 25 µl of the sample and 50 µl water were mixed together and incubated at 37°C for 180 seconds and then the absorbance at 595 nm was taken. The absorbance values were calibrated using BSA as standards.

2.7.5 Measurement of GPX activity.

Samples were homogenised in PBS and centrifuged at 10,000 g for 5 minutes to pellet any cellular debris. The samples were diluted from between 2 to 100 fold to allow accurate measurement. Then 955 µl of reaction mix (5 mg nicotinamide adenine dinucleotide hydrogen phosphate (NADPH), 46 mg reduced glutathione, 3 ml distilled water, 24 ml PBS, 1 ml sodium azide [112.5 mM], and 20 units glutathione reductase), 10 µl sample and 35 µl H_2O_2 (2.2 mM) were added to a cuvette. The rate of change of absorbance was followed at 340 nm (Arthur and Boyne, 1985). A unit of glutathione is defined as that which oxidises 1 µM of NADPH per minute.

2.8 Measurement of selenoproteins.

2.8.1 Determination of selenoprotein profiles.

Profiles of selenoproteins expressed by the various primary skin cells were determined by radio-labelling with [^{75}Se]-selenite as described previously

(Howie *et al*, 1995). Confluent cultures of cells in 75 cm^2 flasks, were labelled with 0.02 MBq/ml of [^{75}Se]-selenite by incubation for 72 hours. Previous studies have shown that [^{75}Se] uptake and incorporation into selenoproteins by cells, reaches a plateau at approximately 24 hours. Therefore cells were labelled for 48-72 hours to ensure a steady state had been reached. Media was decanted and cells were washed twice with EBSS before being harvested into EBSS by scraping, followed by centrifugation at 2000g for 10 minutes. Cells were resuspended in 60 mM Tris-HCl buffer pH 7.8 containing 1 mM DTT and 1 mM EDTA and lysed by sonication. Equal amounts of protein (25 μg) were loaded onto 12% SDS-PAGE gels and proteins were separated by electrophoresis, the gels were dried and the labelled selenoproteins quantified using a molecular phosphoimager, model GS-525 (Biorad, Hemel Hempstead, Herts, UK) and visualised by autoradiography using Kodak X-OMAT XAR-5 film. Selenoprotein profiles were also determined in primary melanocytes, keratinocytes and fibroblasts which had either been pre-labelled with [^{75}Se] for 24 hours prior to being exposed to 200 J/m^2 UVB and then harvested 24 hours post exposure. Alternatively cells were exposed to 200 J/m^2 UVB, then labelled with [^{75}Se] and harvested 24 hours later.

2.8.2 Western blot analysis to identify TR and PHGPX.

Western blot analysis was used to identify TR and PHGPX, the method was carried out by F. Nicol and Dr J. Arthur at the Rowett Research Institute, Aberdeen, UK. The proteins were separated by SDS-PAGE and were transferred to Immobilon polyvinyldifluoride (PVDF) membranes, which were blocked using 10% (v/v) horse serum in 25 mM Tris, 500 mM sodium chloride (pH 7.5) containing 0.05% Tween. The blot was probed with affinity purified polyclonal antibody to rat liver TR at a final dilution of 1:500 (Holmgren and Bjornstedt, 1995) and developed using chemiluminescence or coupled alkaline phosphatase reagents (McLeod *et al*, 1997). The blots were also probed with purified antibodies to rat PHGPX (Roveri *et al*, 1994).

2.9 Statistical Analysis.

The results discussed are from experiments repeated two or three times. Each data point was done in triplicate (numbers of replicates of individual experiments are stated in the text) . Analysis of variance (ANOVA) and the

posthoc Bonferroni correction were used to analyse the significance of the results, values for $P<0.05$ were deemed to be significant. To compare the differences between sodium selenite and selenomethionine ANOVA and the posthoc Scheffe test was used, again values for $P<0.05$ were deemed significant.

Chapter 3

The Effects of Selenium on the Survival and Formation of Lipid Peroxides in UVB Irradiated Skin Cells.

3.1 Introduction.

The cytotoxic effects of UVB radiation on the skin are well documented. UVB radiation can damage cells by numerous means and is far more efficient at causing cell death than UVA. Exposure to UVB can provoke DNA damage, protein cross-linking and the production of free radicals, which themselves can damage DNA and induce lipid peroxidation. Further effects of UVB radiation are discussed in Chapter 1. Cells cultured *in vitro* which are exposed to high doses of UVB radiation and thus receive a high level of damage, exhibit necrotic cell death. However at lower doses of UVB radiation cultured cells have been reported to undergo apoptotic cell death (Henseleit *et al*, 1996). Sunburn cells present in the skin are considered to be severely damaged keratinocytes that are undergoing apoptotic cell death (Bayerl *et al*, 1995), as discussed in detail in Chapter 4. Melanocytes, keratinocytes and fibroblasts have all been proven to be susceptible to cell killing by UVB radiation (Dissanayake *et al*, 1993).

Selenium status and the levels of selenoproteins present in skin cells, appears to be linked with cell susceptibility to damage by UV radiation. The introduction of a GPX-like gene, isolated from the dermatotropic poxvirus *molluscum contagiosum virus*, which resides exclusively in the human epidermis, significantly increased cell survival in cells exposed to UVB (Shisler *et al*, 1998).

It has also been reported that supplementing human skin fibroblasts with sodium selenite (1.2 μM) decreases cell death from 50% to 25%, following exposure to 120 J/m^2 UVB (Richard *et al*, 1990). Similar results using UVA radiation and sodium selenite (1.3 μM) reported that cell death in human skin fibroblasts was decreased from 50% to 10% (Leccia *et al*, 1993). In a

further study supplementation with 320 nM sodium selenite decreased cell death of human skin fibroblasts from 67% to 30% following exposure to UVA (Moysan *et al*, 1995). Moreover Se also decreased the incidence of skin tumours in mice (Overvad *et al*, 1985; Pence *et al*, 1994), and inflammation and sunburn in hairless mice following UVB exposure (Thorling *et al*, 1983). Selenium given both orally and topically increases the minimal erythemal dose (MED) in humans (Burke *et al*, 1992a; Burke *et al*, 1992b), and decreases the number of sunburn cells in human skin following exposure to UVB (La Ruche and Cesarini, 1991), as discussed further in Chapter 1.

Selenium status can increase the abundance of antioxidant selenoenzymes in the skin such as members of the GPX and TR families. The catalytic functions and regulation of the selenoproteins GPX and TR have been discussed in detail in Chapter 1. However in brief, the GPX family of proteins can detoxify a wide range of lipid peroxides and hydrogen peroxide to produce water and the corresponding alcohol's in the presence of reduced glutathione (Roetruck *et al*, 1973). Changes in TR expression may have many effects on the cell including altered growth characteristics or changes in the ability to detoxify free-radicals. In conditions of Se deficiency, decreased levels of GPX and TR could diminish the cells capacity to inactivate ROS. The selenoprotein TR can detoxify lipid hydroperoxides and hydrogen peroxide. Furthermore thioredoxin reductase has been reported to be located on the surface of keratinocytes were it is in an ideal position to detoxify reactive species (reviewed in Schallreuter and Wood, 1989).

Antioxidants other than selenoenzymes can protect human keratinocytes *in vitro* from UVB-induced cell death and apoptosis. The antioxidant α-tocopherol decreases UVB-induced cell death in keratinocytes from 80% to 20% and apoptosis from 80% to 60% (Malorni *et al*, 1996; Kondo *et al*, 1990). Furthermore ascorbic acid also decreases cell death in HaCaT cells (Savini *et al*, 1998). In this context it is clear that UVB exposure produces ROS in the skin. Therefore antioxidants such as selenoproteins can prevent ROS-induced damage to skin cells by inactivating these molecules.

Several techniques have been utilised in this chapter to study cell growth and survival. Including the use of a coulter counter to measure total cell number, therefore allowing the measurement of cell growth following Se

supplementation in all cell types. The trypan blue assay was employed to measure: selenium toxicity, cell survival following UVB irradiation, cell survival following Se treatment and exposure to UVB radiation and cell death following menadione treatment in keratinocytes and melanocytes. The trypan blue dye exclusion method was utilised as live cells exclude dye and dead cells take up the dye. This phenomenon allows the % cell death to be calculated. Finally, for the fibroblast experiments the colony formation assay was used as it was found that once a toxic insult was applied to the fibroblasts any dead cells would adhere to the tissue culture plastic and could not be removed by trypsin. Therefore to correct for the false survival rates found with the trypan blue assay, the colony formation assay was used.

One of the mechanisms by which exposure to UVB can damage skin cells is as mentioned previously, by the production of ROS. Reactive oxygen species can elicit peroxidation reactions in cell membrane lipids (Punnonen *et al*, 1991). Reactive oxygen species are difficult to measure directly as they are very short lived, therefore it is common to measure their presence by the formation of secondary products (reviewed in Rumley and Paterson, 1998). Polyunsaturated fatty acids in the plasma membrane are one of the cellular targets of ROS. The most common by-product of lipid peroxidation is malondialdehyde (MDA), it arises predominantly from the oxidation of polyunsaturated fatty acids with three or more double bonds. Malondialdehyde is formed following exposure of cells to UVB in a dose-dependant manner (Stewart *et al*, 1996). Exposure to UVA also causes MDA formation in a dose-dependent manner (Moysan *et al*, 1995). Reactive oxygen species remove the hydrogen atom from the methylene bridges of unsaturated fatty acids, resulting in lipid hydroperoxides. In the presence of transition metals, the lipid hydroperoxides then break down to form aldehydes, including MDA.

The most widely utilised technique for measuring MDA production is the thiobarbituric acid (TBA) reaction or "TBAR assay". During the TBAR assay MDA reacts with thiobarbituric acid giving rise to a MDA-TBA adduct (Asakawa and Matsushita, 1979; Wasowicz *et al*, 1993). The MDA-TBA adduct can be measured by using a spectrophotometer (Lefevre *et al*, 1996), fluorimeter (Conti *et al*, 1991) or more specifically by high-performance liquid chromatography (Wong *et al*, 1987). However, other substances can

also react with TBA such as other lipid peroxides, amino acids and sugars. Consequently, the term thiobarbituric acid reactive substances (TBARS) is used to describe the results more accurately (Bonnefont *et al*, 1989). Although the measurement of TBARS is not a direct measure of MDA, it is widely used to measure lipid peroxidation of membranes (Moysan *et al*, 1993; Kobayashi *et al*, 1996a). Due to a lack of reproducibility with the TBAR assay, a commercial assay was utilised in this study to measure MDA. Whilst the commercial assay displays less cross-reactivity with other substances and the product formed is more stable than the TBA-MDA adduct, it works by the same principles as the TBAR assay (Fig 2.3).

Lipid peroxidation is inhibited by the use of free radical scavengers, including topically applied glutathione, which diminishes the formation of TBARS in irradiated mouse skin (Kobayashi *et al*, 1996b). Furthermore ascorbic acid injected intraperitoneally or intracutaneously decreases the formation of TBARS in mouse skin following exposure to UVB (Kobayashi *et al*, 1996a). Finally vitamin E added immediately after exposure to UVB also decreases the formation of MDA in human skin fibroblasts by at least 50% (Kondo *et al*, 1990).

Selenium also decreases the formation of TBARS in patients. Selenium (200 μg/day) given prior to patients exposure to a solar simulator, decreased the level of TBARS formed by 13% (Pietschmann *et al*, 1992). Furthermore cultured fibroblasts given sodium selenite (320 nM) supplementation for 3 days prior to exposure to UVA, displayed a decrease of 50% in the formation of TBARS (Moysan *et al*, 1995; Leccia *et al*, 1993).

Most of the experiments in the present study have been carried out using primary keratinocytes. Keratinocytes are the most numerous cell type in the epidermis, they receive the highest exposure to UVB and they are the cell type that forms basal and squamous cell carcinomas. Basal and squamous cell carcinomas are accepted to be induced by exposure to UVB radiation (Stenbach, 1975; reviewed in Elmets and Mukhtar, 1996). A limited number of studies have been performed on melanocytes and fibroblasts.

Throughout this project two types of Se compounds were used. Selenomethionine which is an organic form of Se, is widely used in

supplementation studies as it is the form of Se most commonly available in the diet. Sodium selenite was also used, it is an inorganic form which again has been widely used in experiments involving Se supplementation. Sodium selenite is very reactive and at high concentrations can become an oxidant itself. These two compounds are described and discussed in Chapter 1.

The aims of this chapter were -:

- To study the effects of sodium selenite and selenomethionine supplementation on cell growth and survival, of primary human skin cells.

- To investigate if sodium selenite and selenomethionine supplementation protects human primary melanocytes, fibroblasts and keratinocytes *in vitro* from UVB-induced cell death.

- To ascertain the mechanism by which Se exerts its protective effect.

3.2 Methods and Results.

3.2.1 Effect of Se supplementation on cell growth and cell viability.

Cells were grown (Chapter 2, section 2.2) in six-well dishes till 70% confluent, then treated with sodium selenite or selenomethionine (1 nM-10 μM) and incubated for a further 72 hours. The toxicity of the compounds were determined by cell viability counts using trypan blue exclusion (Chapter 2, section 2.3.1). Growth stimulation was assessed by counting cells using a Coulter counter (Chapter 2, section 2.3.2).

Fibroblasts at a logarithmic growth phase were seeded out at $1x10^3$ in petri dishes. The fibroblast were allowed to attach to the dishes for 24 hours, before Se was added to the media and the cells, incubated for a further 72 hours. The media was then replaced with fresh media and the cells incubated for 7-14 days. The colonies of cells were visualised with crystal violet (Chapter 2, section 2.3.3).

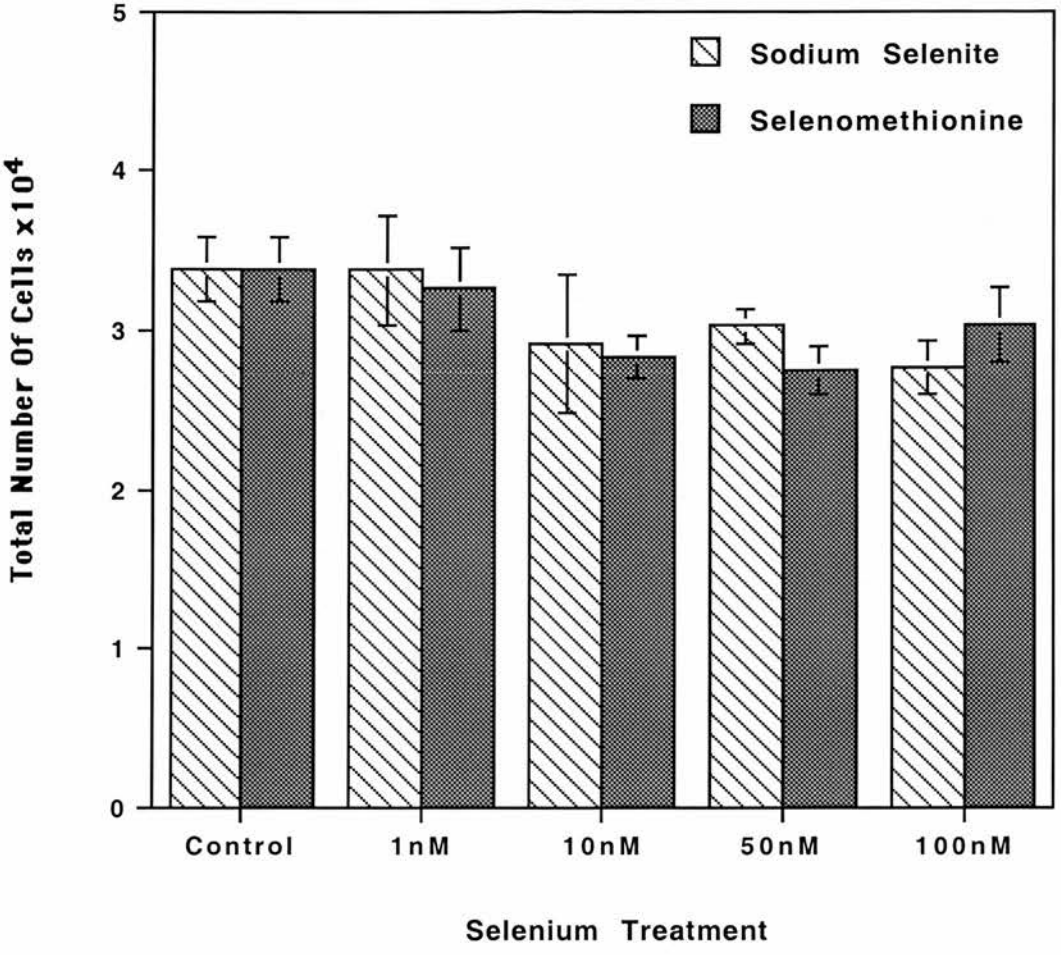
Primary human keratinocytes.
Treatment of primary keratinocytes with sodium selenite or selenomethionine had no apparent effect on growth (Fig 3.1a). Sodium selenite was toxic at concentrations of 1 μM (24% cell death) or above with 56% cell death found at 10 μM (Fig 3.1b). Selenomethionine was not found be toxic at concentrations up to 10 μM (Fig 3.1b).

Primary human melanocytes.
Similar results were found in primary melanocytes in that, sodium selenite and selenomethionine had no growth stimulating activity (Table 3.1a) and selenomethionine was found to be non-toxic up to 10 μM. Sodium selenite however was found to be toxic at 10 μM (44% cell death) (Table 3.1b).

HaCaT cells.
Neither of the Se compounds induced growth in HaCaT cells (Table 3.2a). However, HaCaT cells were found to be more susceptible to the cytotoxicity of the Se compounds, sodium selenite was toxic to the cells even at 200 nM *

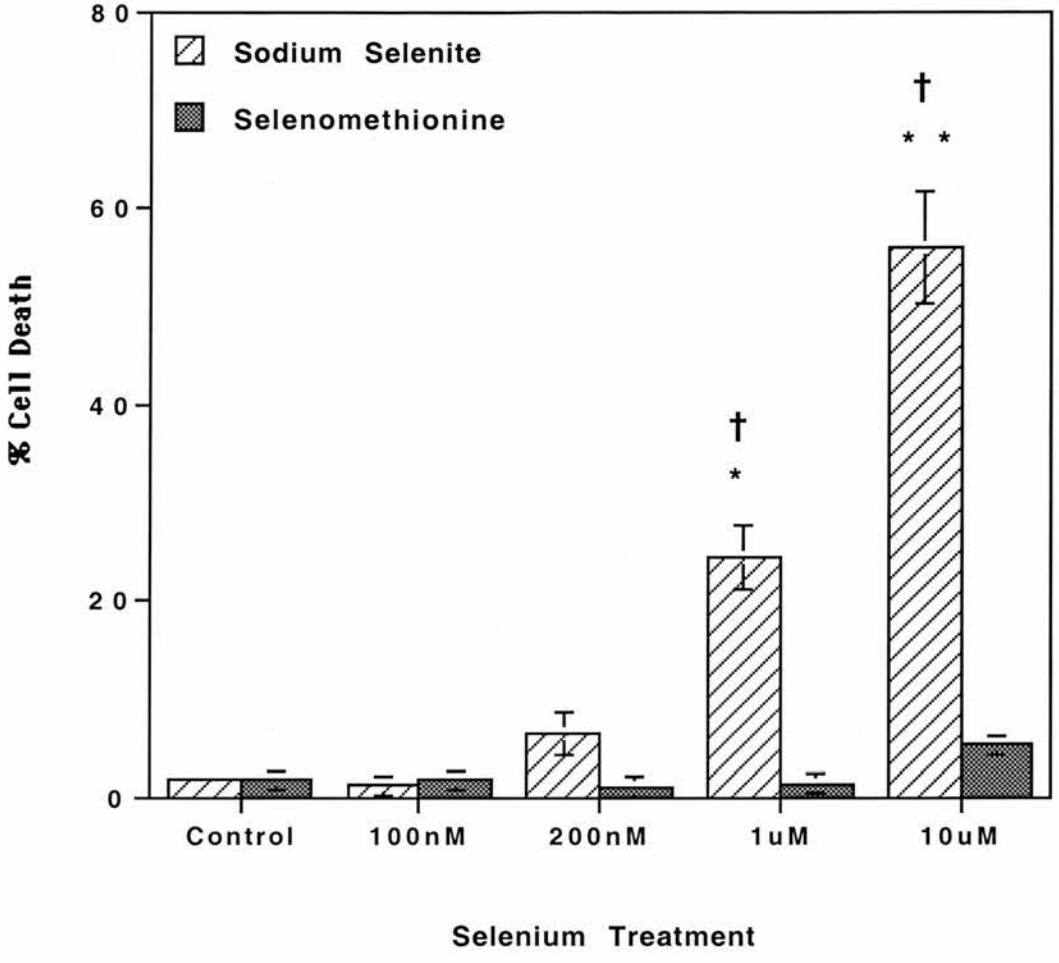
Continued on page 3-12

Figure 3.1a: Effect of sodium selenite or selenomethionine on the growth of primary human keratinocytes.



Primary human keratinocytes were incubated with sodium selenite or selenomethionine for 72 hours, and the cell growth was measured by counting the cells using a Coulter counter. Control cells had no Se added to them. Results are the means of total number of cells ± S.E.M, n=3. No significant differences from control.

Figure 3.1b: Effect of sodium selenite or selenomethionine on the viability of primary human keratinocytes.



Primary human keratinocytes were incubated with sodium selenite or selenomethionine for 72 hours, and the viable cells counted using a haemocytometer and trypan blue exclusion. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Control cells had no Se added to them. Significant differences from control, * = $P<0.05$, ** = $P<0.01$. † = significant differences between the sodium selenite and selenomethionine, $P<0.05$.

Table 3.1a: Effect of sodium selenite or selenomethionine on the growth of primary human melanocytes.

	Total no of cells(10^4) ± S.E.M.	
Dose(nM)	Sodium selenite	Selenomethionine
Control	1.32±0.65	1.32±0.65
1	1.44±0.31	1.34±0.07
2	1.32±0.21	1.58±0.02
10	1.44±0.02	1.29±0.08
100	1.31±0.15	1.52±0.11

Primary human melanocytes were incubated with sodium selenite or selenomethionine for 72 hours, and the total number of cells counted using a Coulter counter. Control cells had no Se added to them. Results are the means of the total number of cells ± S.E.M, n=3. No significant differences from control.

Table 3.1b: Effect of sodium selenite or selenomethionine on the viability of primary human melanocytes.

	% Cell Death ±S.E.M.	
Dose(μM)	Sodium selenite	Selenomethionine
Control	2.9±1.3	2.9±1.3
0.1	3.2±0.9	3.1±1.2
0.2	4.5±2.3	2.3±0.1
1	5.1±2.2	4.3±0.9
10	†44.9±5.3**	7.3±0.4

Primary melanocytes were incubated with sodium selenite or selenomethionine for 72 hours, and the viable cells counted using a haemocytometer and trypan blue exclusion. Control cells had no Se added to them. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Significant difference from control, * = $P<0.05$, ** = $P<0.01$. † = Significant differences between sodium selenite and selenomethionine treated cells, $P<0.05$.

Table 3.2a: Effect of sodium selenite or selenomethionine on the growth of HaCaT cells.

	Total no of cells(10^4) ± S.E.M.	
Dose(nM)	Sodium selenite	Selenomethionine
Control	2.38±0.25	2.38±0.25
1	2.22±0.33	2.14±0.08
2	2.13±0.50	2.39±0.06
10	2.39±0.23	2.40±0.09
100	2.40±0.33	2.37±0.16

HaCaT cells were incubated with sodium selenite or selenomethionine for 72 hours, and the total number of cells counted using a Coulter counter. Control cells had no Se added to them. Results are the means of the total number of cells ± S.E.M, n=3. No significant differences from control.

Table 3.2b: Effect of sodium selenite or selenomethionine on the viability of cell line HaCaT.

	% Cell Death ±S.E.M.	
Dose(μM)	Sodium selenite	Selenomethionine
Control	3.8±1.5	3.8±1.5
0.1	5.6±0.3	3.1±0.2
0.2	†19.9±6.4*	2.35±0.1
1	†16.9±1.1*	4.35±0.5
10	†81.9±2.7**	20.8±0.3*

HaCaT cells were incubated with sodium selenite or selenomethionine for 72 hours, and the viable cells counted using a haemocytometer and trypan blue exclusion. Control cells had no Se added to them. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Significant difference from control, * = $P<0.05$, ** = $P<0.01$. † = Significant differences between sodium selenite and selenomethionine treated cells, $P<0.05$.

Table 3.3: Effect of sodium selenite or selenomethionine on the viability of primary human fibroblasts.

	No of Colonies ±S.E.M.	
Dose(μM)	Sodium selenite	Selenomethionine
Control	214±6.3	214±6.3
0.01	198±9.9	188±5.6
0.05	236±10.5	178±11.9
0.1	215±6.9	208±4.6
0.2	†168±8.8*	211±5.6
1	†154±12.3*	186±14.2
10	†113±11.2**	221±12.3

Primary human fibroblasts were incubated with sodium selenite or selenomethionine for 72 hours, the media was then replaced and the cells left until colonies had formed on the control plates (7-14 days). Control cells had no Se added to them. Plates were then stained with crystal violet for 1 minute and the number of colonies with over 25 cells counted on an image analysis system. Results are the means of total number of colonies ± S.E.M, n=3. Significant difference from control, * = $P<0.05$, ** = $P<0.01$. † = Significant differences between sodium selenite and selenomethionine treated cells, $P<0.05$.

with 19.9% cell death induced, 20% cell death was observed at 1 μM, which increased at 10 μM to 81% (Table 3.2b). Selenomethionine was also found to be slightly toxic towards HaCaT cells with 20% cell death at 10 μM.

Primary human fibroblasts.
The experiments on fibroblasts differ from the other cell types as they were done using the colony formation assay. However neither Se compound stimulated the growth of fibroblasts (Table 3.3). Sodium selenite was shown to be significantly toxic at concentrations of 200 nM and upwards, but selenomethionine was not toxic at any of the concentrations measured (Table 3.3).

3.2.2 Induction of cell death by exposure to UVB radiation.

To determine the lethal UVB dose for the various cell types, cells were grown to 70% confluence, the media was removed and retained, then the monolayer was covered with PBS. The cells were irradiated with broadband UVB (Chapter 2, section 2.2.6). To determine the UVB dose that killed between 50-80% of the cells, doses of between 480-1440 J/m^2 were given. The original media was replaced and cell viability was determined by cell counts on a haemocytometer using trypan blue (0.4% w/v) exclusion (Chapter 2, section 2.3.1), 24 and 48 hours after irradiation.

For the fibroblast experiments cells in a logarithmic growth phase were seeded out at a density of $1x10^3$ in petri dishes. The fibroblast were allowed to attach for 24 hours, then the media was replaced with PBS and the cells were exposed to UVB (100-400 J/m^2). The original media was then returned and the cells were incubated for 7-14 days. The colonies were visualised with crystal violet (Chapter 2, section 2.3.3).

Primary human keratinocytes.
Primary keratinocytes were found to be relatively resistant to cell death induced by exposure to UVB radiation. The rate of cell death was found to be very low at 24 hours (5-25%) however this increased greatly at 48 hours post exposure to UVB radiation (Fig 3.2a). The dose of UVB which killed 65% of primary human keratinocytes at 48 hours was around 960 J/m^2 (Fig 3.2a).

Primary human melanocytes.

Primary melanocytes were more susceptible to cell death induced by UVB radiation compared to primary keratinocytes, again the rate of cell death was higher at 48 hours (Fig 3.2b). The dose required to kill 56% of the cells at 48 hours was 720 J/m^2.

HaCaT cells.

HaCaT cells were also susceptible to cell death induced by exposure to UVB, with more (25-90%) cell death at 24 hours than that observed in keratinocytes (5-25%) and melanocytes (5-50%). However the dose required to kill 69% of cells at 48 hours was 960 J/m^2 (Fig 3.2c), which was similar to the primary keratinocytes (65%) (fig 3.2a).

Primary human fibroblasts.

Fibroblast experiments were carried out using the colony formation assay. The colony formation assay measures the effect of UVB radiation, on cell division, therefore the results can not be directly compared to those with trypan blue. However the results do demonstrate that the amount of UVB required to stop cell division by 50% is 170 J/m^2 (Fig 3.2d).

3.2.3 Effect of Se pre-treatment on UVB-induced cell death.

Cells cultured in six-well dishes until 70% confluent were treated with sodium selenite or selenomethionine (1 nM-1 μM), and incubated for 24 hours. The media was removed and set aside and 1ml of PBS added to each well immediately prior to the cells being exposed to UVB. Typically the cells were exposed to UVB for 1.5-2 minutes (720-960 J/m^2; the dose given depended on the cell type used), which resulted in approximately 70-80% cell death 48 hours post irradiation, in cultures where no sodium selenite or selenomethionine had been added. After UVB exposure, PBS was removed and the original culture media was replaced. Viability was determined by trypan blue exclusion 48 hours post UVB exposure (Chapter 2, section 2.3.1).*

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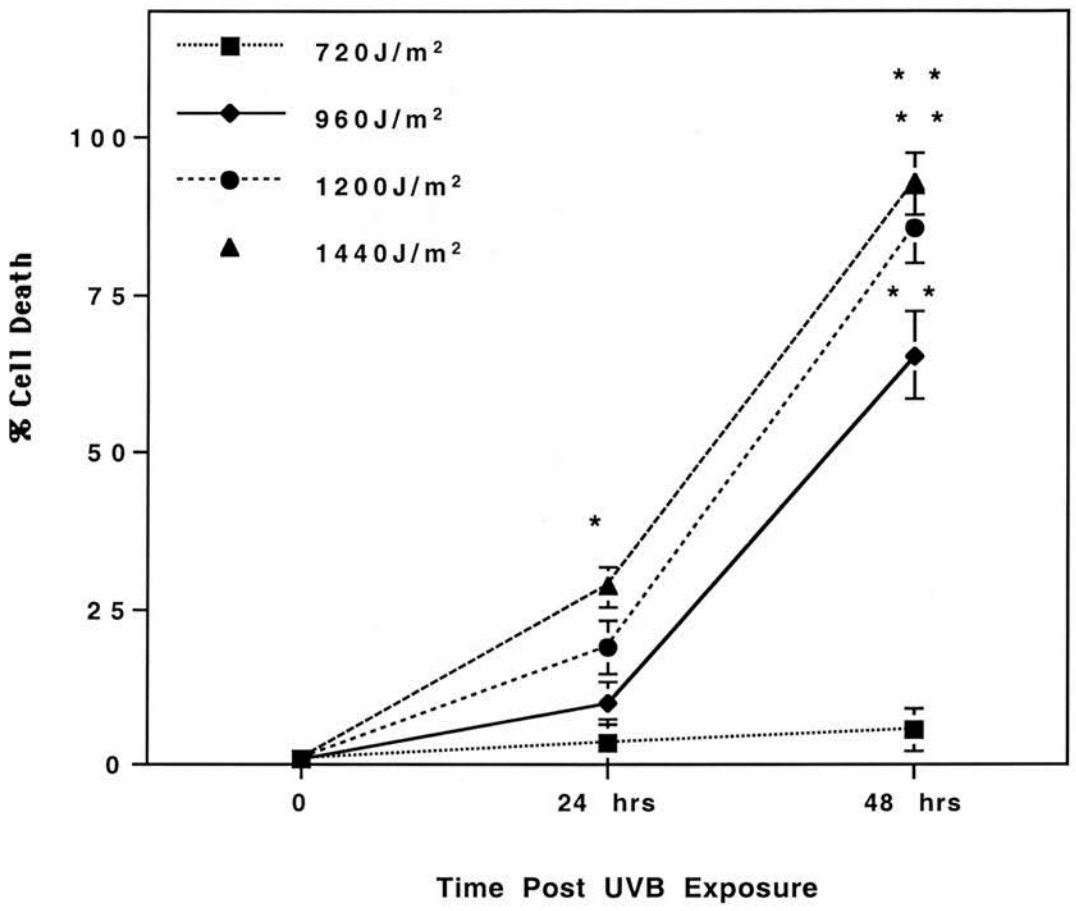
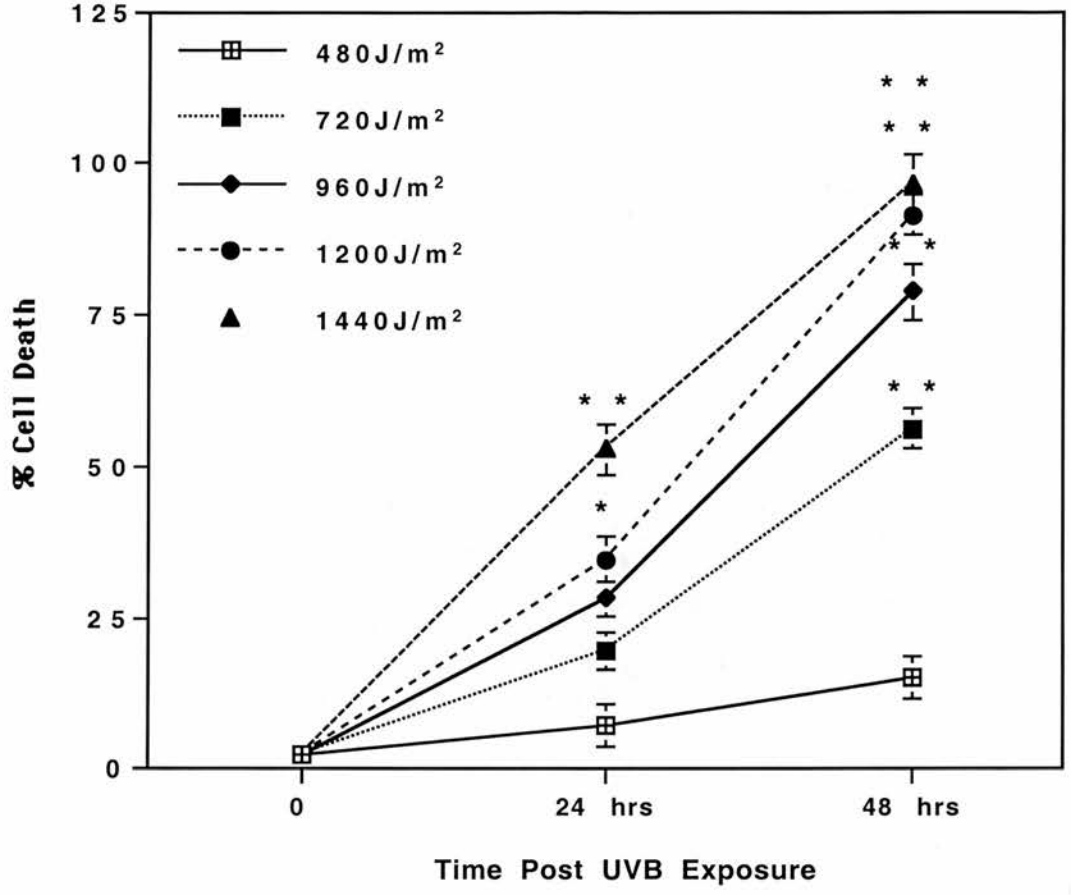
Figure 3.2a: Effect of increasing UVB exposure on the viability of primary human keratinocytes.



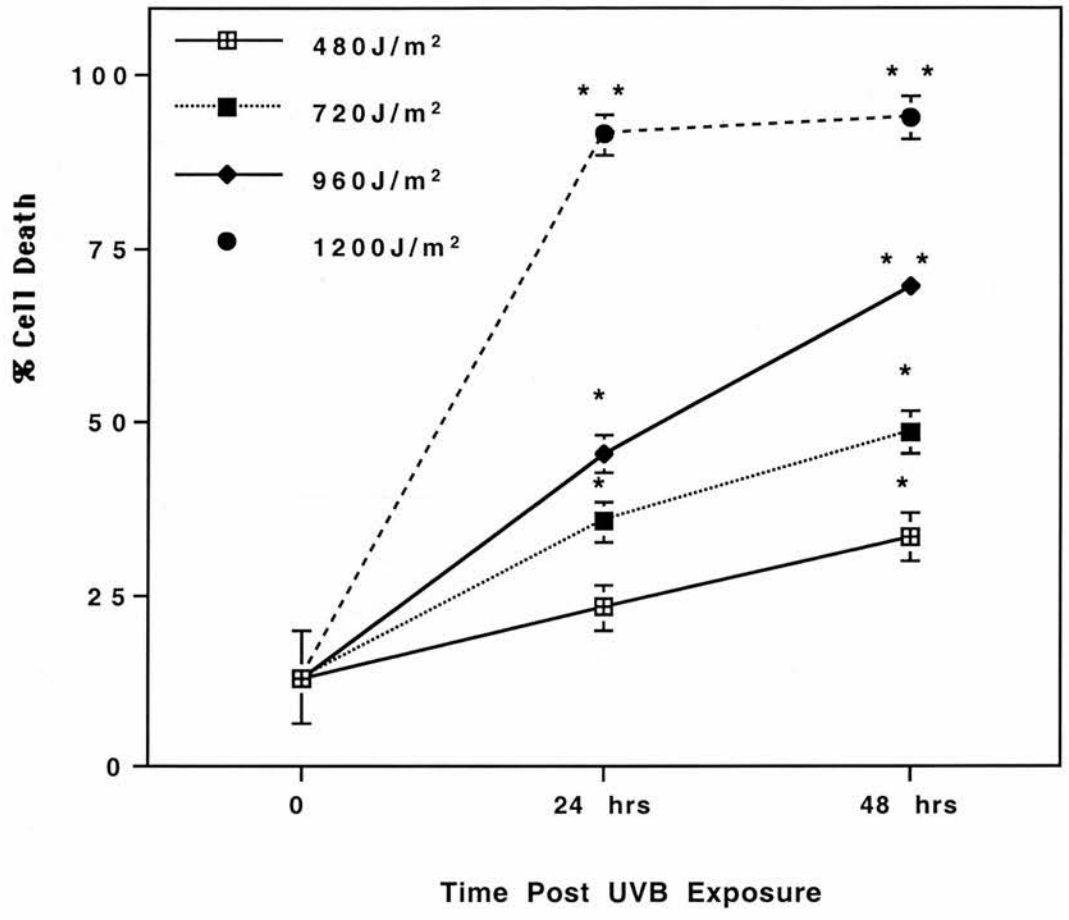
Media was removed from primary keratinocytes and replaced with PBS prior to exposure to various doses of UVB radiation. The original media was then replaced and the cells incubated for 24 or 48 hours before cell counts were performed using a haemocytometer and trypan blue exclusion. Control cells received no UVB. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Significant differences from cells not exposed to UVB, * = $P<0.05$, ** = $P<0.01$.

Figure 3.2b: Effect of increasing UVB exposure on the viability of primary human melanocytes.



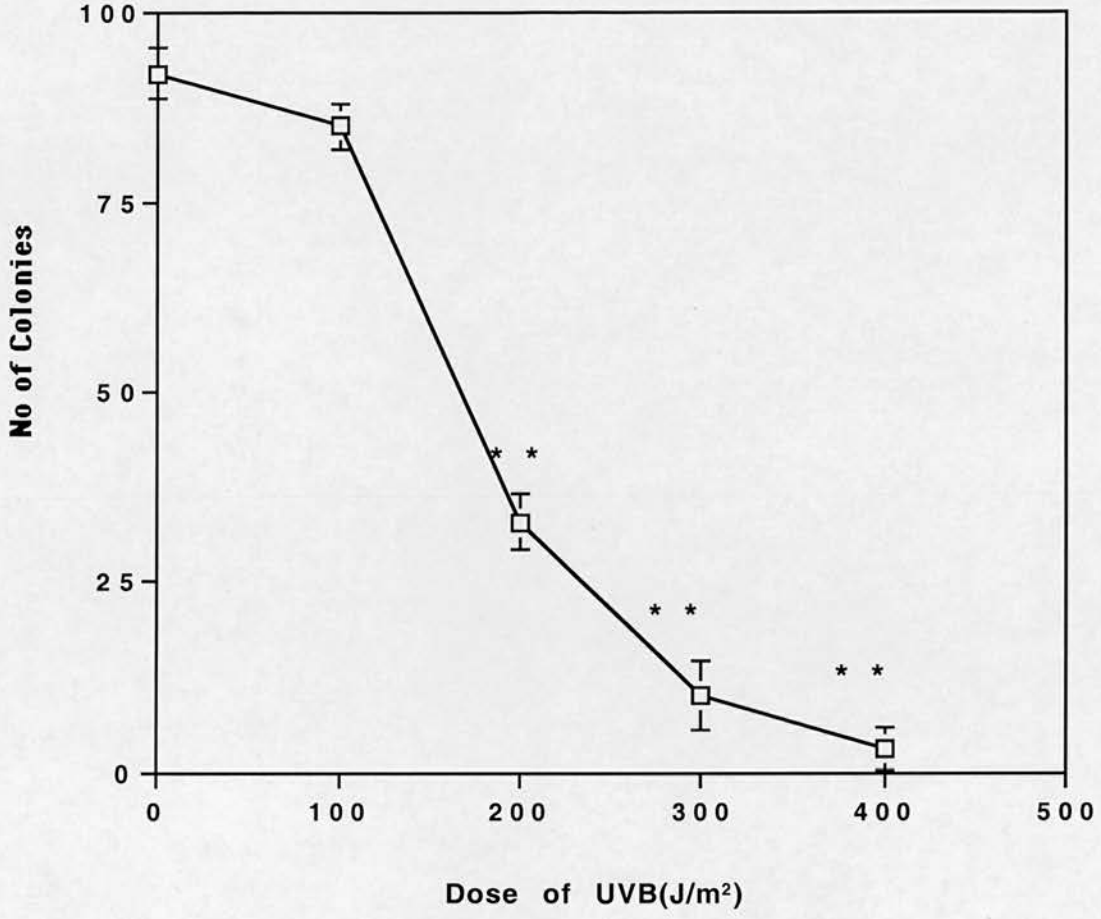
Media was removed from primary melanocytes and replaced with PBS prior to exposure to various doses of UVB radiation. The original media was then replaced and the cells incubated for 24 or 48 hours before cell counts were performed using a haemocytometer and trypan blue exclusion. Control cells received no UVB. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Significant differences from cells not exposed to UVB, * = $P<0.05$, ** = $P<0.01$.

Figure 3.2c: Effect of increasing UVB exposure on the viability of HaCaT cells.



Media was removed from the HaCaT cells and replaced with PBS prior to exposure to various doses of UVB radiation. The original media was then replaced and the cells incubated for 24 or 48 hours before cell counts were performed using a haemocytometer and trypan blue exclusion. Control cells received no UVB. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Significant differences from cells not exposed to UVB, * = $P<0.05$, ** = $P<0.01$.

Figure 3.2d: Effect of increasing UVB exposure on the viability of primary human fibroblasts.



Media was removed from the primary fibroblasts and replaced with PBS prior to exposure to various doses of UVB radiation. The original media was then replaced and the cells left until colonies had formed on the control plates (7-14 days). Plates were then stained with crystal violet for 1 minute, and the number colonies with over 25 cells counted on an image analysis system. Control cells received no UVB. Results are the means of total number of colonies ± S.E.M, n=3. Significant differences from control, * = $P<0.05$, ** = $P<0.01$.

For the fibroblast and one set of keratinocyte experiments, cells in a logarithmic growth phase were plated out at $1x10^3$ in petri dishes. The cells were allowed to attach for 24 hours, sodium selenite or selenomethionine was then added to the media for 24 hours. The media was then replaced with PBS and the cells were exposed to UVB (100-400 J/m^2). The original Se-containing media was then returned and the cells were incubated for 7-14 days. The colonies were visualised with crystal violet (Chapter 2, section 2.3.3).

Primary human keratinocytes.

The dose of UVB used (960 J/m^2) produced 76% cell death in primary keratinocytes 48 hours after exposure (Fig 3.3a), which is slightly higher than for the previous experiment (65%) (Fig 3.2a). The differences in the levels of cell death provoked by UVB maybe due to donor to donor variation. The addition of sodium selenite or selenomethionine 24 hours prior to exposure to UVB, prevented a marked amount of cell death, but the protective effects were significantly different between the two compounds (Fig 3.3a). A protective effect with sodium selenite was seen with concentrations from 1-200 nM, with an optimal protective concentration of 50 nM. Selenomethionine was less potent than sodium selenite in protecting cells from UVB-induced damage, with higher concentrations of selenomethionine compared to sodium selenite required to produce significant protection from UVB-induced damage. No significant protective effect of selenomethionine was observed below a concentration of 10 nM. Maximal protection using selenomethionine was found at 50 nM, with concentrations in the range of 10 nM-1 μM providing significant protection. As with sodium selenite, the protection afforded by selenomethionine started to diminish at 1 μM.

The experiments on primary keratinocytes were also repeated using the colony formation assay with similar results. The optimal protective concentrations of sodium selenite were 10-100 nM which was a greater range than observed in the trypan blue experiments (Fig 3.3b). With selenomethionine the optimal protective concentration was also 10-100 nM with the protective range of 10-200 nM. Finally the protective effect was lost at 200 nM sodium selenite, which was a lower concentration than that observed in the trypan blue experiment and at 1 μM selenomethionine which is the same concentration as seen in the trypan blue experiments.

Experiments were also carried out in which sodium selenite or selenomethionine was omitted from the culture media after exposure to UVB. Protection from cell death following UVB exposure was still achieved. Furthermore the protection appeared to be almost identical to that found when Se was present for the duration of the experiment (Figs 3.3a and 3.4). Maximal protection was achieved with Se concentrations in the pre-treated media of 50-200 nM for sodium selenite or selenomethionine. Concentrations of Se in the range of 1 nM-1 μM of sodium selenite and 50 nM-10 μM selenomethionine offered significant protection.

Primary melanocytes.

Melanocytes were more sensitive to UVB than keratinocytes, and a UVB dose of 720 J/m^2 was sufficient to kill 82% of the cells (Table 3.4). Again the level of cell death was slightly higher than the previous experiment (56%), however this may be due to inter-donor differences (Fig 3.2b). In the experiments with melanocytes, the effects of Se were similar to those seen in keratinocytes with maximal protection being achieved for sodium selenite and selenomethionine at concentrations of 10 nM and 100 nM respectively and little or no protection found at 1 μM (Table 3.4). Concentrations of Se in the range of 1-200 nM sodium selenite and 10 nM-1 μM selenomethionine offered significant protection.

HaCaT cells.

In the keratinocyte cell line HaCaT, a dose of 960 J/m^2 UVB was required to kill approximately 80% of the cells (Table 3.4). Again the amount of cell death at this dose of UVB was higher than found previously (70%), this is due to inter-experimental differences (Fig 3.2c). The maximal protection provided by Se was found at 10 nM and 100 nM sodium selenite and selenomethionine respectively (Table 3.4). Concentrations in the range of 1-200 nM sodium selenite and 10 nM-200 nM selenomethionine offered significant protection.

Primary fibroblasts.

Fibroblasts also exhibited a reduction in cell death stimulated by UVB exposure following pre-treatment with Se compounds. This effect was less *

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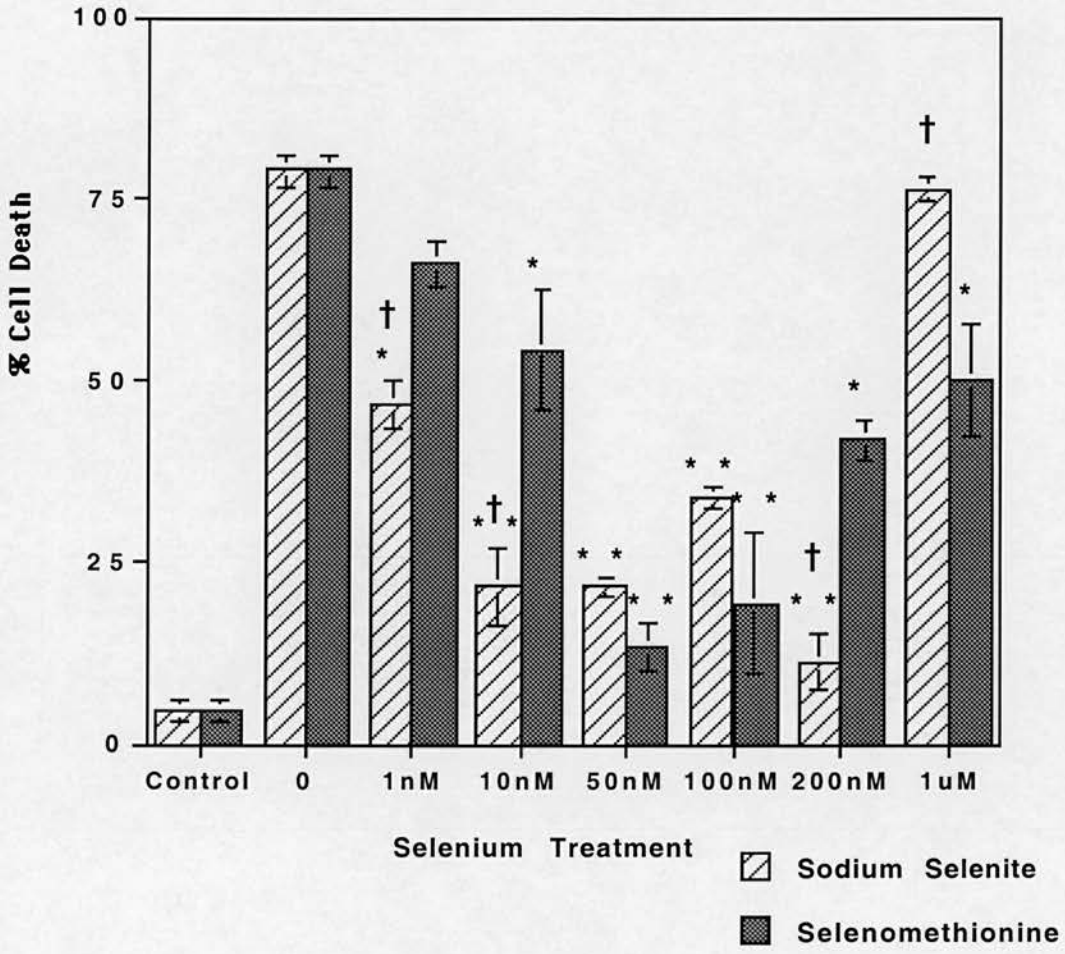
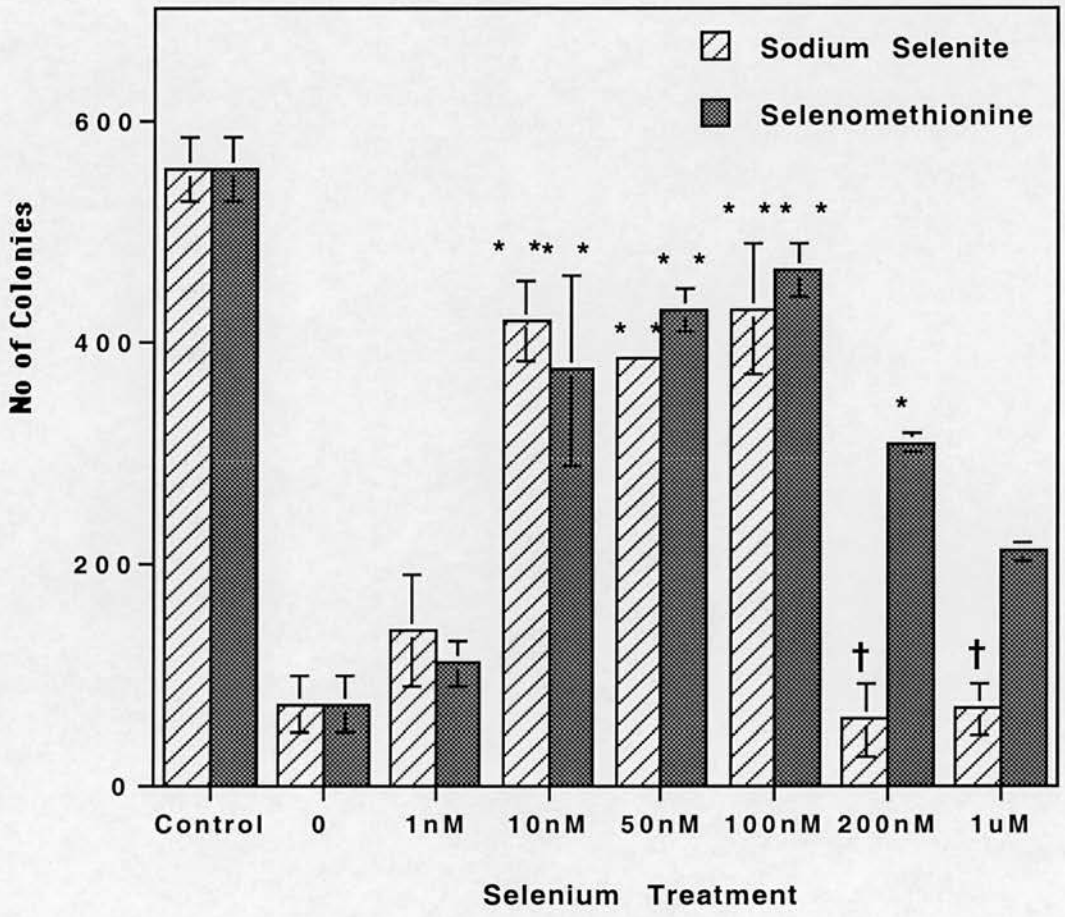
Figure 3.3a: The effect of selenomethionine or sodium selenite pre-treatment on the viability of primary human keratinocytes after exposure to UVB.



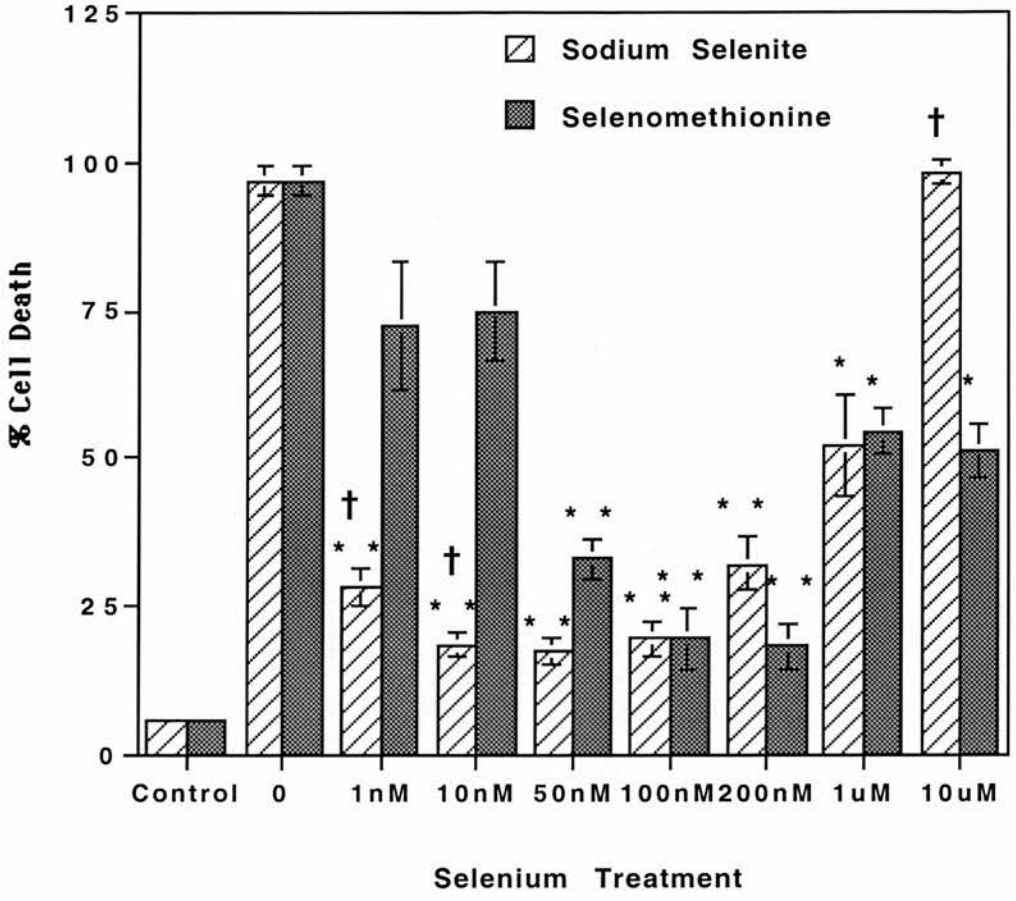
Primary keratinocytes were pre-treated with sodium selenite or selenomethionine for 24 hours prior to the media being replaced with PBS and the cells exposed to UVB (960 J/m^2). The original Se-containing media was returned and the cells incubated for 48 hours before cell counts were performed using a haemocytometer and trypan blue exclusion. Control cells received no UVB or Se supplement. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Significant differences from cells not treated with Se, but exposed to UVB, * = $P<0.05$, ** = $P<0.01$. † = Significant differences between sodium selenite and selenomethionine, $P<0.05$.

Figure 3.3b: The effect of selenomethionine or sodium selenite pre-treatment on the viability of primary human keratinocytes after exposure to UVB, measured by the colony formation assay.



Primary keratinocytes were pre-treated with sodium selenite or selenomethionine for 24 hours prior to the media being replaced with PBS and the cells exposed to UVB (200 J/m^2). The original Se-containing media was returned and the cells left until colonies had formed on the control plates (7-14 days). Plates were then stained with crystal violet for 1 minute and the number of colonies with over 25 cells were counted on an image analysis system. Results are the means of % cell death ± S.E.M, n=3. Significant differences from cells not treated with Se, but exposed to UVB, * = $P<0.05$, ** = $P<0.01$. † = Significant differences between sodium selenite and selenomethionine, $P<0.05$.

Figure 3.4: The effect of selenomethionine or sodium selenite pre-treatment only on the viability of primary human keratinocytes after exposure to UVB.



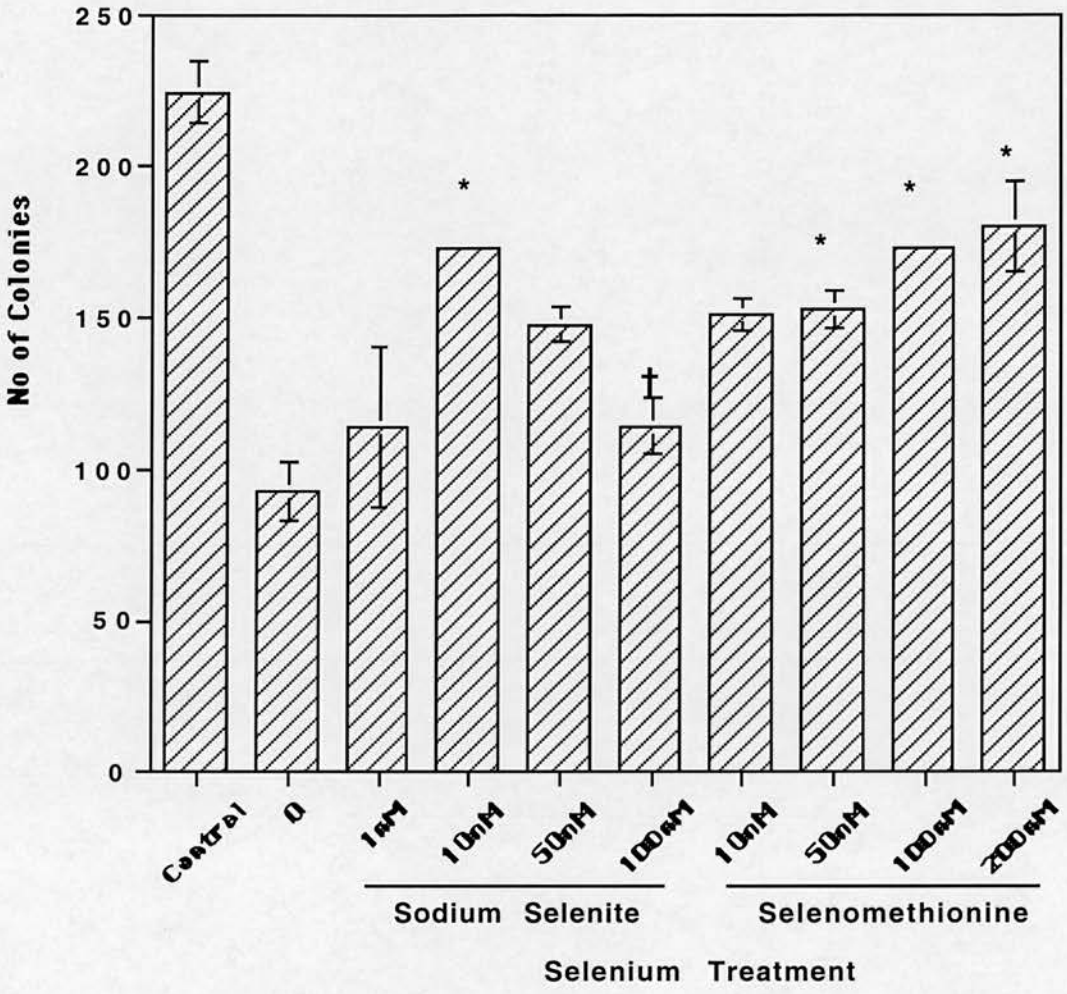
Primary keratinocytes were pre-treated with sodium selenite or selenomethionine for 24 hours prior to the media being replaced with PBS and the cells exposed to UVB (960 J/m^2). The original media was replaced with media from cells of the same passage, with no Se added. Then incubated for 48 hours before cell counts were performed using a haemocytometer and trypan blue exclusion. Control cells received no UVB or Se supplement. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Significant differences from cells not treated with Se, but exposed to UVB, * = $P<0.05$, ** = $P<0.01$. † = Significant differences between sodium selenite and selenomethionine, $P<0.05$.

Table 3.4: The effect of selenomethionine or sodium selenite pre-treatment on the viability of primary human melanocytes and the human keratinocyte cell line HaCaT after exposure to UVB.

	% Cell Death ±S.E.M.			
	Sodium selenite		Selenomethionine	
Dose (nM)	Melanocytes	HaCaT's	Melanocytes	HaCaT's
Control	7.7±1.6	4.3±4.3	7.7±1.6	4.3±4.3
0	82.6±1.4	86.8±1.1	82.6±1.4	86.8±1.1
1	†57.7±6.4*	†57.6±2.9*	79.3±1.7	79.4±3.8
10	†10.3±0.9**	†19.5±1.4**	72.4±2.3*	64.3±2.2*
50	22.5±1.5**	35.4±1.9**	26.9±1.5**	23.8±4.5**
100	†29.2±1.1**	†45.2±2.8*	14.3±4.9**	11.6±3.9**
200	34.4±3.5**	†60.4±2.2*	30.3±3.4**	39.5±3.1**
1000	75.4±3.4	86.7±2.3	65.6±3.3*	75.2±3.4

Primary melanocytes and HaCaT cells were pre-treated with sodium selenite or selenomethionine for 24 hours prior to the media being replaced with PBS and the cells exposed to UVB (melanocytes 720 J/m^2 and HaCaT cells 960 J/m^2). The original Se-containing media was returned and the cells incubated for 48 hours before cell counts were performed using a haemocytometer and trypan blue exclusion. Control cells received no UVB or Se supplement. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Significant differences from cells not treated with Se, but exposed to UVB, * = $P<0.05$, ** = $P<0.01$. † = Significant differences between sodium selenite and selenomethionine, $P<0.05$.

Figure 3.5: The effect of selenomethionine or sodium selenite pre-treatment on the viability of primary human fibroblasts after exposure to UVB.



Primary fibroblast were pre-treated with sodium selenite or selenomethionine for 24 hours prior to the media being replaced with PBS and the cells exposed to UVB (150 J/m^2). The original Se-containing media was returned and the cells incubated until colonies had formed on the control plates (7-14 days). Plates were then stained with crystal violet for 1 minute and the number of colonies with over 25 cells were counted on an image analysis system. Control cells received no UVB or Se supplement. Results are the means of total number of colonies ± S.E.M, n=3. Significant differences from cells not treated with Se, but exposed to UVB, * = $P<0.05$, ** = $P<0.01$. † = Significant differences between sodium selenite and selenomethionine, $P<0.05$.

than that measured in other skin cell types (Fig 3.5). Exposure to UVB decreased colony formation by 55% and sodium selenite protected against the reduction in colony formation by 24% to 79%. Selenomethionine proved more consistent than sodium selenite at protecting the cells. Significant increases in colony formation were found with concentrations of 50-200 nM. The optimal concentration was 200 nM with, a 25% increase in colony formation.

3.2.4 Effect of Se being added post UVB exposure on UVB-induced cell death.

In a separate set of experiments primary keratinocytes were grown and irradiated as in section 3.2.3, however before irradiation, no Se was added to the media. Selenium was only added following exposure to UVB and the cells were incubated for 48 hours and then counted using trypan blue staining as in section 3.2.3.

Protection was still achieved, but the protection was modest and required higher concentrations of Se compared to protection achieved by adding sodium selenite or selenomethionine to cells 24 hours prior to UVB exposure (Fig 3.6). Maximal protection was achieved at 50 nM and 200 nM for sodium selenite and selenomethionine respectively. The level of protection was diminished with only a 2-3 fold decrease in cell death for the cells treated with Se post-UVB. In comparison there was a 5-6 fold decrease in death in the cells pre-treated with Se for 24 hour (Fig 3.3a and 3.6). Sodium selenite was more effective in protecting cells with concentrations in the range of 10 nM-1 μM offering modest but significant protection, compared to 100-200 nM for selenomethionine.

3.2.5 Absorbance spectrum of sodium selenite and selenomethionine.

Sodium selenite and selenomethionine were dissolved in PBS at concentrations of 100 mM. The absorbance of the two solutions was then measured on a spectrophotometer using a solution of PBS alone to measure the background absorbance.*

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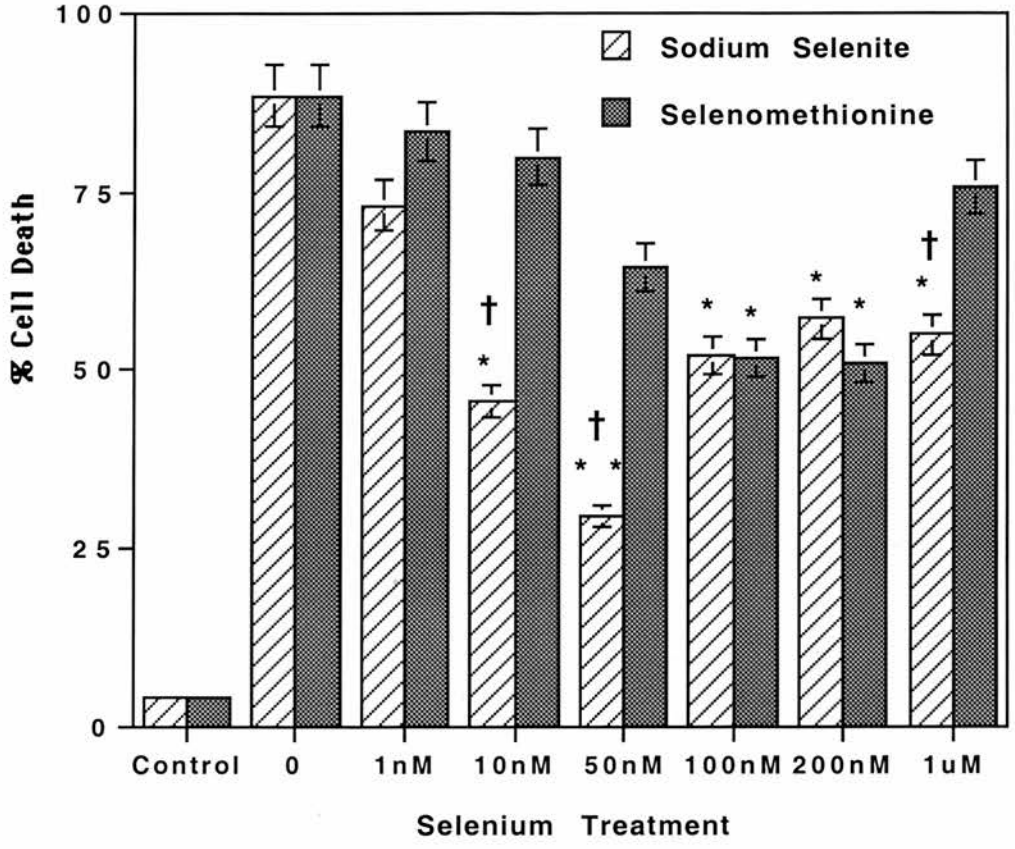
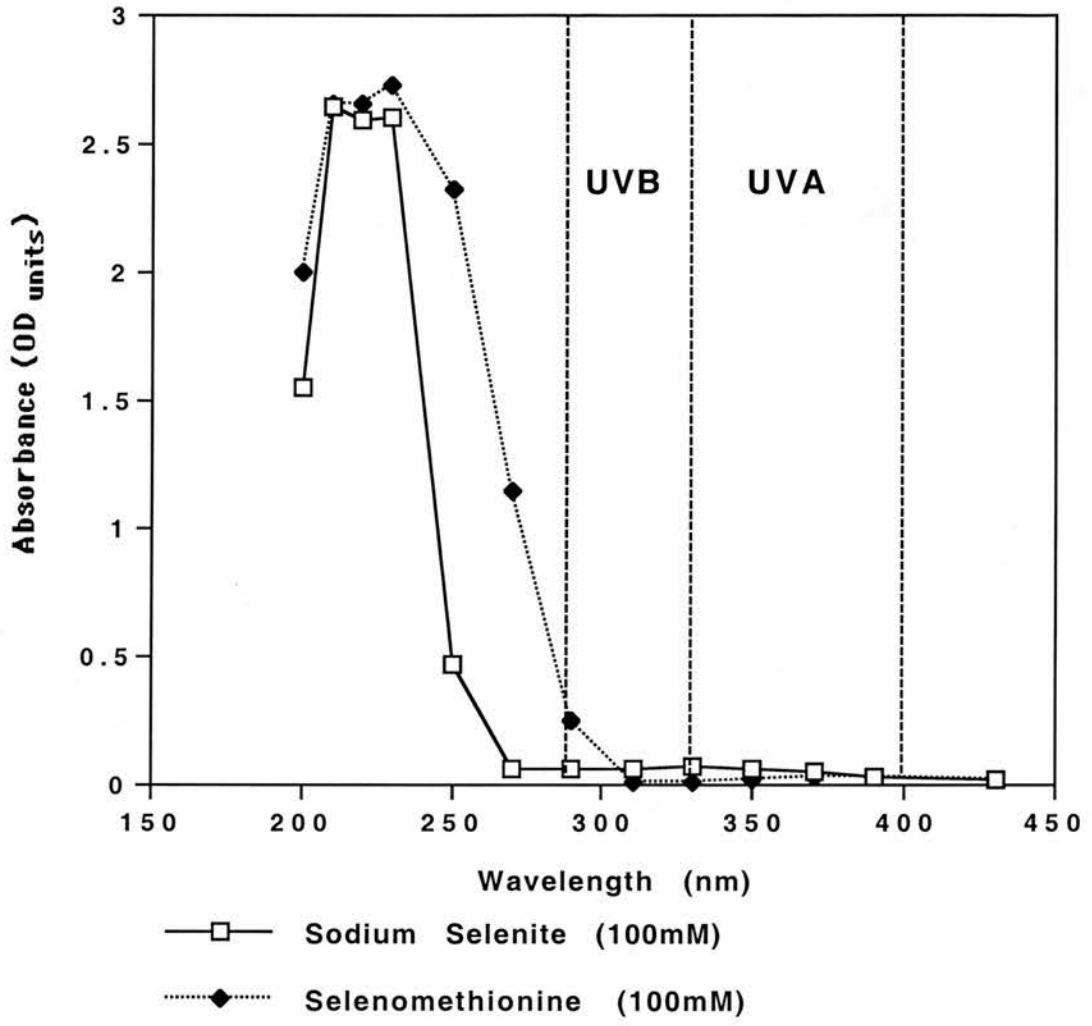
Figure 3.6: The effect of sodium selenite or selenomethionine added after the exposure of primary human keratinocytes to UVB.



The media on primary keratinocytes was replaced with PBS, prior to the cells being exposed to UVB (960 J/m^2). The original media was returned, with either sodium selenite or selenomethionine added and the cells were incubated for 48 hours before cell counts were performed using a haemocytometer and trypan blue exclusion. Control cells received no UVB or Se supplement. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Significant differences from cells not treated with Se, but exposed to UVB, * = $P<0.05$, ** = $P<0.01$. † = Significant differences between sodium selenite and selenomethionine, $P<0.05$.

Figure 3.7: The absorbance spectrums of 100 mM solutions of sodium selenite or selenomethionine.



Absorbance of 100 mM solutions of selenomethionine and sodium selenite were measured on a spectrophotometer. Both chemicals were dissolved in PBS and the absorption was measured against a PBS only reference.

Figure 3.7 demonstrates that 100mM solutions of sodium selenite and selenomethionine dissolved in PBS had no absorption within the UVA spectrum. Selenomethionine had a slight absorbance in the UVB spectrum and sodium selenite had none. They both have a high absorption within the UVC spectrum. (Fig 3.7).

3.2.6 Effect of varying the pre-incubation times of the Se compounds prior to UVB exposure.

Primary keratinocytes were treated as in section 3.2.3, however instead of a 24 hour pre-incubation with Se, the Se was added to the cells between 3-48 hours prior to the cells exposure to UVB. The cells were then exposed to UVB, conditioned media without Se was replaced on the cells and 48 hours later the cells were counted by trypan blue exclusion as in section 3.2.3.

Varying the time that the Se is on the cells prior to UVB exposure, shows that both Se compounds have to be present on the cells for at least 24 hours, for significant protection to be demonstrated (Fig 3.8). Sodium selenite started to offer the cells protection at 12 hours pre-treatment, compared to 24 hours for selenomethionine. The protection increased at 48 hours for both compounds.

3.2.7 Effect of the addition of cycloheximide and Se pre-treatment on UVB-induced cell death.

Again primary keratinocytes were treated as in section 3.2.2, however at the same time that the Se was added to the cell media, the protein synthesis inhibitor cycloheximide (10 $\mu g/ml$) was added. Therefore the cells were pre-treated for 24 hours prior to exposure to UVB with Se and cycloheximide. The keratinocytes were exposed to a lower dose of UVB (600 J/m^2), and fresh conditioned media without Se or cycloheximide was returned to the cells. The cells were counted by trypan blue exclusion 48 hours post exposure to UVB.

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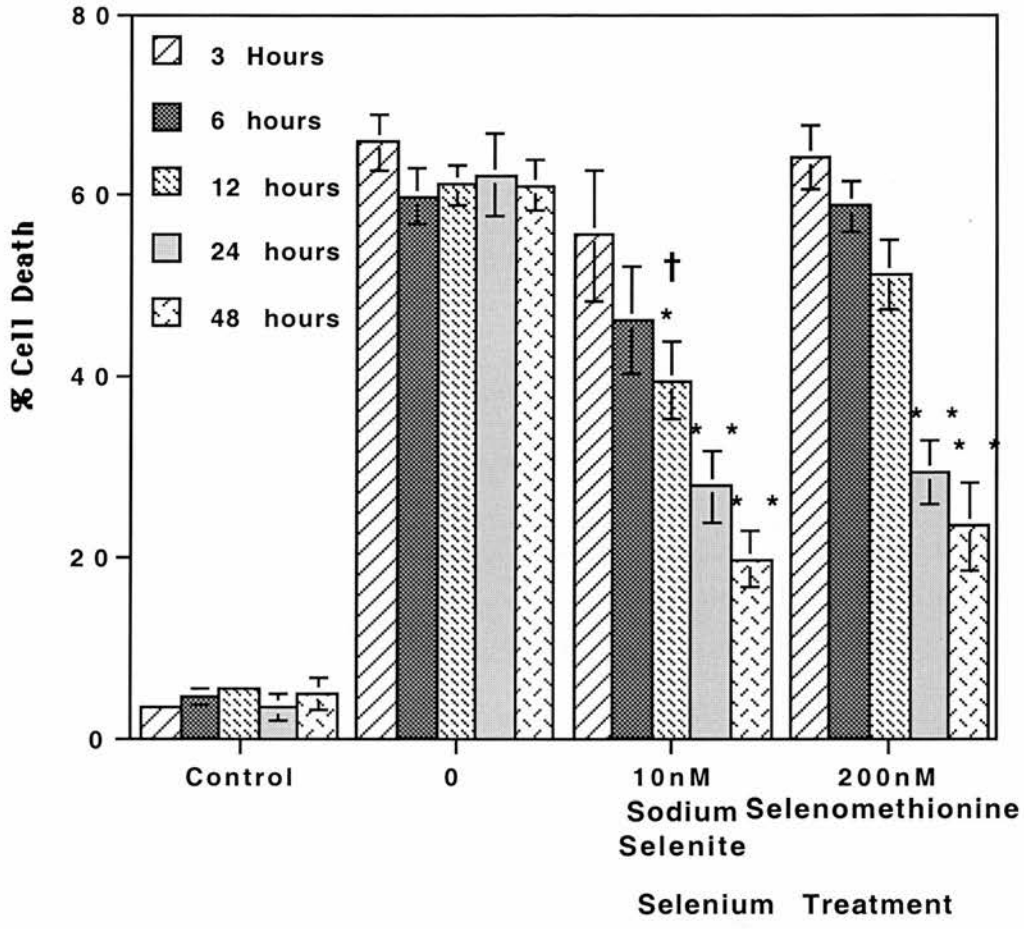
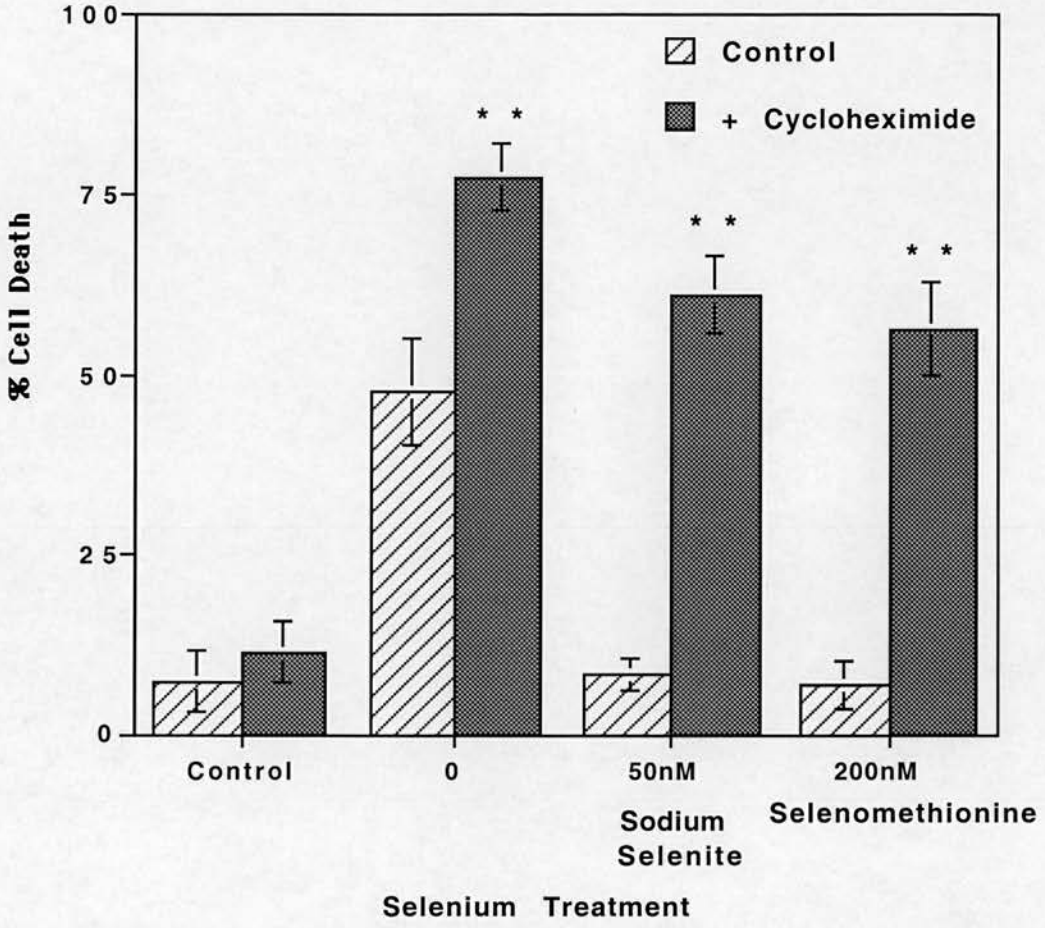
Figure 3.8: The effect of sodium selenite or selenomethionine added at various times to primary human keratinocytes prior to their exposure to UVB.



Sodium selenite or selenomethionine was added to the media of primary keratinocytes at various time points prior to the media being replaced with PBS, and the cells exposed to UVB (960 J/m^2). The original media was replaced with conditioned media with no Se, and the cells were incubated for 48 hours, before cell counts were performed using a haemocytometer and trypan blue exclusion assay. Control cells received no UVB or Se supplement. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Significant differences from cells not treated with Se, but exposed to UVB, * = $P<0.05$, ** = $P<0.01$. † = Significant differences between sodium selenite and selenomethionine, $P<0.05$.

Figure 3.9: The effect of sodium selenite or selenomethionine, and cycloheximide added to primary human keratinocytes prior to their exposure to UVB.



Sodium selenite or selenomethionine and cycloheximide were added to the media of primary keratinocytes 24 hours prior to the media being replaced with PBS, and the cells exposed to UVB (600 J/m^2). Fresh conditioned media (with no Se or cycloheximide) was returned to the cells, and the cells were incubated for 48 hours before cell counts were performed using a haemocytometer and trypan blue exclusion. Control cells received no UVB or Se supplement. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Significant differences from cells not treated with cycloheximide, but exposed to UVB and Se, * = $P<0.05$, ** = $P<0.01$.

Cycloheximide was utilised to prevent synthesis of selenoproteins. After exposure to UVB, fresh conditioned media was returned to the cells without Se or cycloheximide. The removal of cycloheximide was to allow the cells to produce heat shock proteins and mount a normal cellular response to UVB. However, as demonstrated in Fig 3.9 this does not appear to have occurred. Even although a lower dose of UVB was used (600 J/m^2), the cells exposed to UVB and cycloheximide display a higher level of cell death 78% compared to 48% in cells only exposed to UVB.

3.2.8 Effect of high concentration selenomethionine.

Cells were treated as in section 3.2.1, however higher levels of selenomethionine were used (10 μM-1 mM) and cell viability was measured by trypan blue exclusion.

Higher concentrations of selenomethionine were employed to attempt to answer the question of why higher concentrations of selenomethionine lose their protective effect. To investigate if the selenomethionine was at sub-toxic concentrations, higher levels were given to cells. Selenomethionine was found to be toxic to primary keratinocytes only at 1 mM and on primary melanocytes at 50 μM (Fig 3.10a and 3.10b). Both of these concentrations are 50-1000 times more concentrated than the levels used in the protection studies.

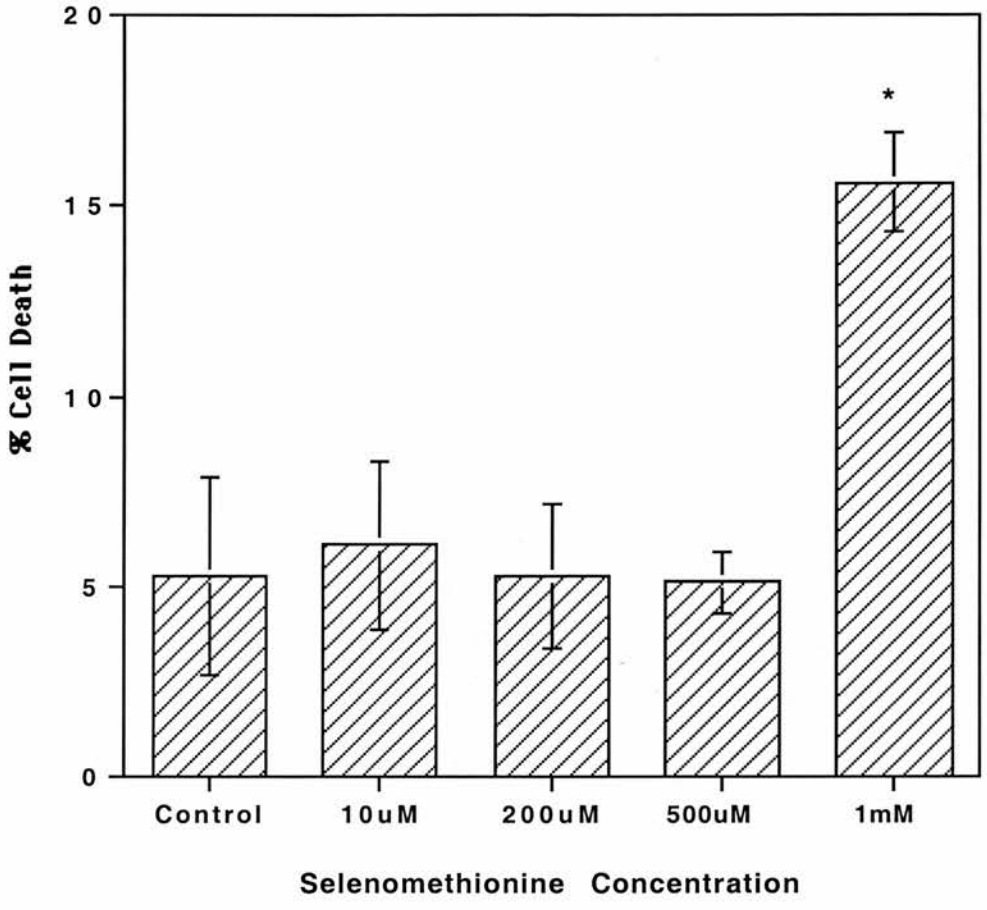
3.2.9 Effect of UVB-irradiated sodium selenite or selenomethionine on primary keratinocytes.

Selenomethionine and sodium selenite dissolved in PBS were exposed to 1000 J/m^2 UVB. The toxicity of the irradiated solution were compared to unirradiated solutions. The toxicity was compared by treating primary keratinocytes with the solutions for 72 hours. The cells were then counted by trypan blue exclusion as in section 3.2.1.

*

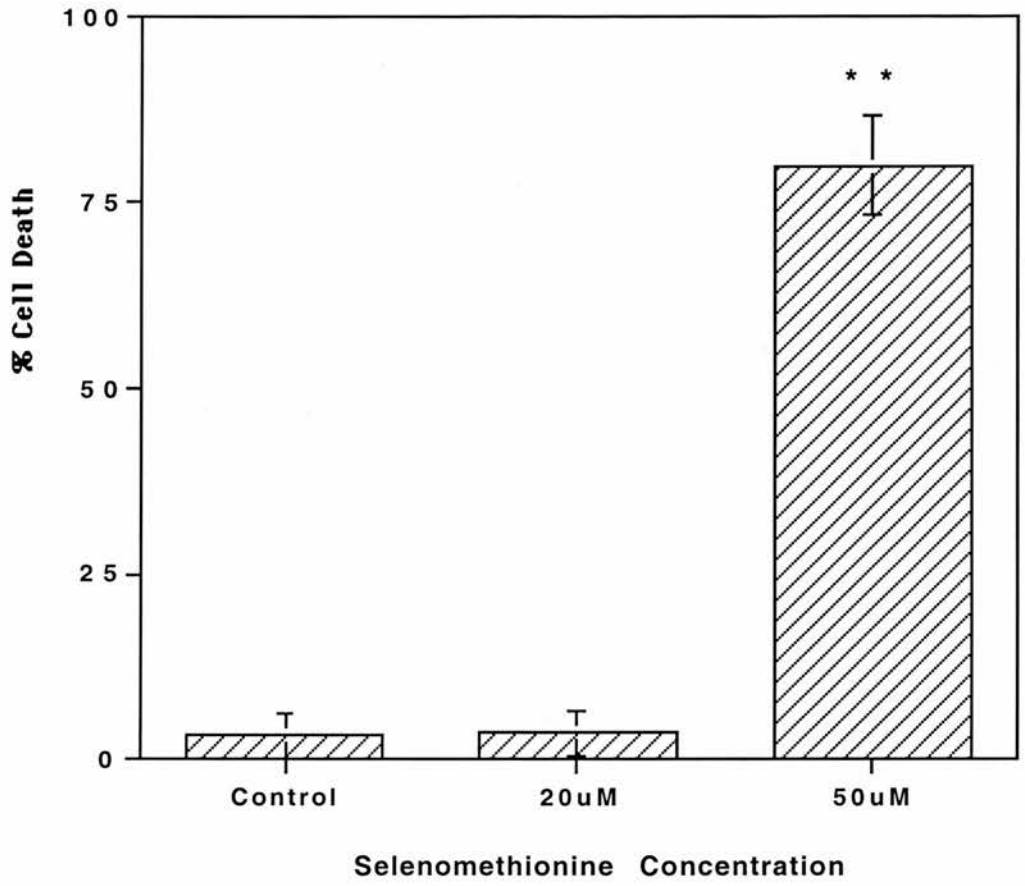
Continued on page 3-35

Figure 3.10a: Effect of high levels of selenomethionine on the viability of primary human keratinocytes.



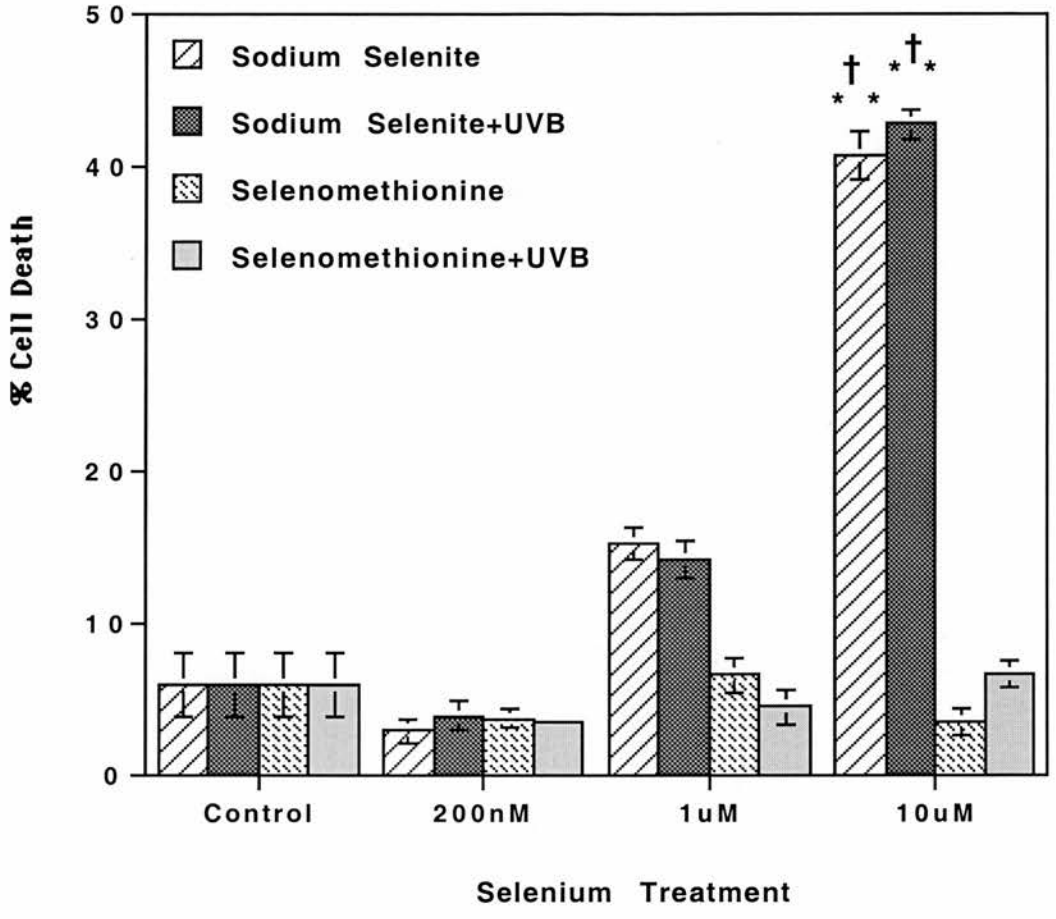
Primary human keratinocytes were incubated with selenomethionine for 72 hours, and the viable cells counted using a haemocytometer and trypan blue exclusion. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Control cells had no Se added to them. Results are the means of % cell death ± S.E.M, n=3. Significant differences from control, * = $P<0.05$, ** = $P<0.01$.

Figure 3.10b: Effect of high levels of selenomethionine on the viability of primary human melanocytes.



Primary human melanocytes were incubated with selenomethionine for 72 hours, and the viable cells counted using a haemocytometer and trypan blue exclusion. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Control cells had no Se added to them. Significant differences from control, * = $P<0.05$, ** = $P<0.01$.

Figure 3.11: Effect of irradiated sodium selenite or selenomethionine on the viability of primary human keratinocytes.



Primary human keratinocytes were incubated with irradiated sodium selenite or selenomethionine (1000 J/m^2) for 72 hours, and the viable cells counted using a haemocytometer and trypan blue exclusion. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Control cells had no Se added to them. Significant differences from control, * = $P<0.05$, ** = $P<0.01$. † = Significant differences between sodium selenite and selenomethionine, $P<0.05$.

It was found that pre-irradiation did not alter the toxicity of the Se solutions (Fig 3.11).

3.2.10 Effect of Se pre-treatment on menadione-induced cell death.

To demonstrate if the protective effect of selenomethionine was lost at high concentrations with other toxic assaults, menadione was utilised. This compound induces oxidative stress in cells. First the level of menadione to induce cell death in keratinocytes was investigated. In the literature, cells are usually pulsed with menadione for 2-4 hours to induce cell death, therefore it was decided to use a 3 hour treatment. Cells were cultured to 70% confluence and then pulsed for 3 hours with various concentrations of menadione made in PBS. The cells were then washed well with PBS and fresh media returned, 48 hours later the cell viability was counted using trypan blue exclusion.

Preliminary experiments indicated that menadione was non-toxic at 50 μM, but was toxic at 100 μM, killing most of the cells (98%) (Fig 3.12). Subsequent experiments were performed by pulsing the cells for 3 hours with 100 μM menadione to induce cell death.

Primary keratinocytes were treated as above, however the cells were also pre-treated for 24 hours with sodium selenite or selenomethionine. The cells were then exposed to menadione and survival quantitated by trypan blue exclusion.

Selenium treatment for 24 hours prior to exposure to menadione protected the cells from cell death (Fig 3.13). Sodium selenite was markedly better at affording this protection than selenomethionine. Sodium selenite was protective at 10-100 nM, much the same concentrations (1-200 nM, Fig 3.3a) as when UVB is the toxic insult. Selenomethionine was found to be protective at 50 nM only, unlike when UVB was used, where concentrations of 10nM-1 μM offered protection (Fig 3.3a). The protection was lost at higher concentrations of Se, much like with UVB response. However, the level of protection was lower than that seen using UVB exposure. *

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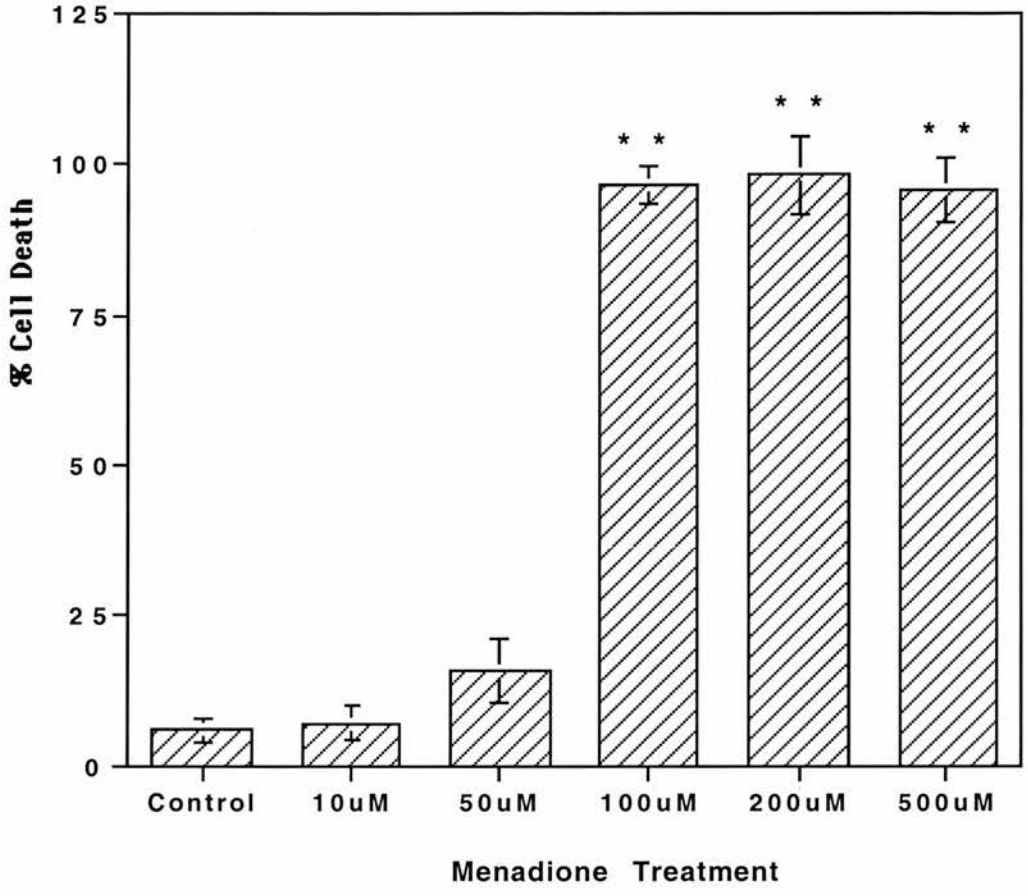
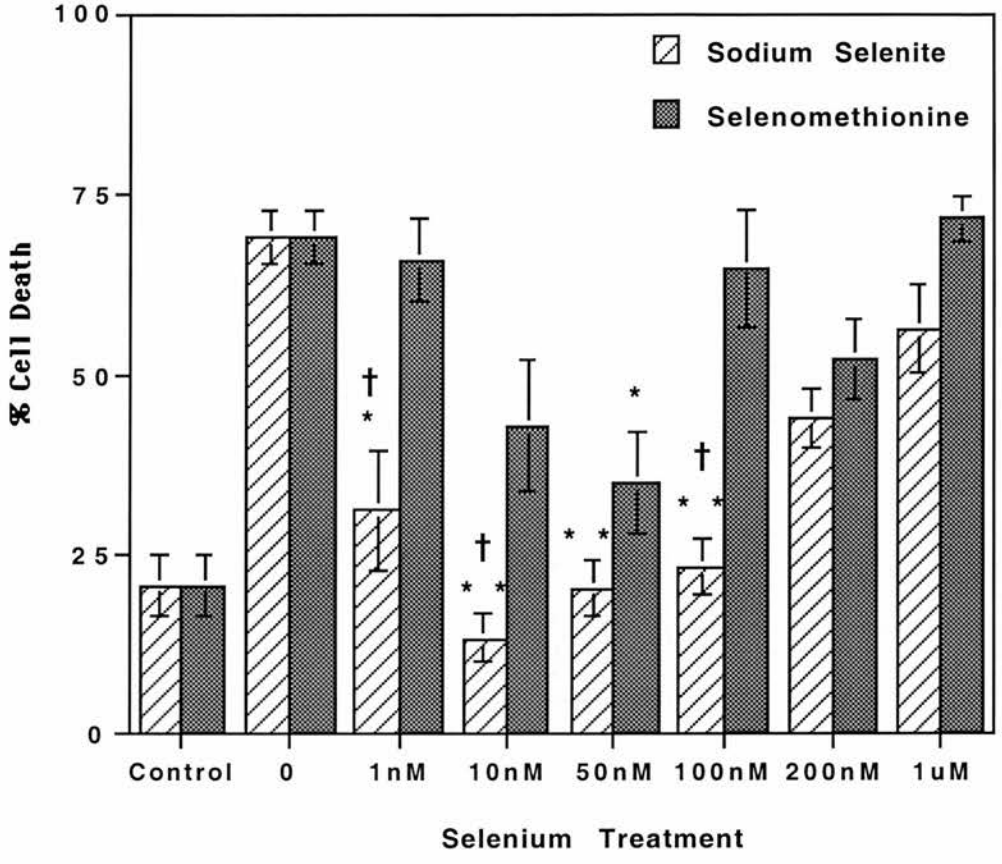
Figure 3.12: Effect of menadione on the viability of primary human keratinocytes.



Primary human keratinocytes were incubated with menadione prepared in media for 3 hours, then washed with PBS and fresh media returned. The cells were then left for 48 hours, and the viable cells counted using a haemocytometer and trypan blue exclusion. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Control cells had no menadione added to them. Significant differences from control, * = $P<0.05$, ** = $P<0.01$.

Figure 3.13: The effect of selenomethionine or sodium selenite pre-treatment on the viability of primary human keratinocytes after exposure to menadione.



Primary keratinocytes were pre-treated with sodium selenite or selenomethionine for 24 hours prior to the media being replaced with media containing 100 μM menadione for 3 hours. The original media was returned and the cells incubated for 48 hours before cell counts were performed using a haemocytometer and trypan blue exclusion. Control cells received no menadione or Se treatment. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Significant differences from cells not treated with Se, but exposed to UVB, * = $P<0.05$, ** = $P<0.01$. † = Significant differences between sodium selenite and selenomethionine, $P<0.05$.

3.2.11 Effect of increasing the level of UVB exposure to cells pre-treated with Se.

Primary keratinocytes were treated as in section 3.2.3, however increasing doses of UVB were given to the cells.

When the level of UVB that the cells were exposed to was increased, the protective effect of the Se was gradually lost. The protection is displayed in Fig 3.14. When the UVB dose was over 1000 J/m^2 protection diminished when using both compounds. However below 1000 J/m^2 protection was afforded by both seleno-compounds.

3.2.12 Effect of UVB and Se on the formation of MDA.

HaCaT cells were grown in petri dishes until they were 70% confluent, the media was replaced with PBS and the cells were exposed to 1000 J/m^2 UVB. The original media was returned to the cells and they were incubated for 0-24 hours following exposure to UVB. The cells were then harvested and the levels of MDA measured using a commercial assay (Calbiochem, Nottingham). The method is described further in Chapter 2, section 2.3.4.

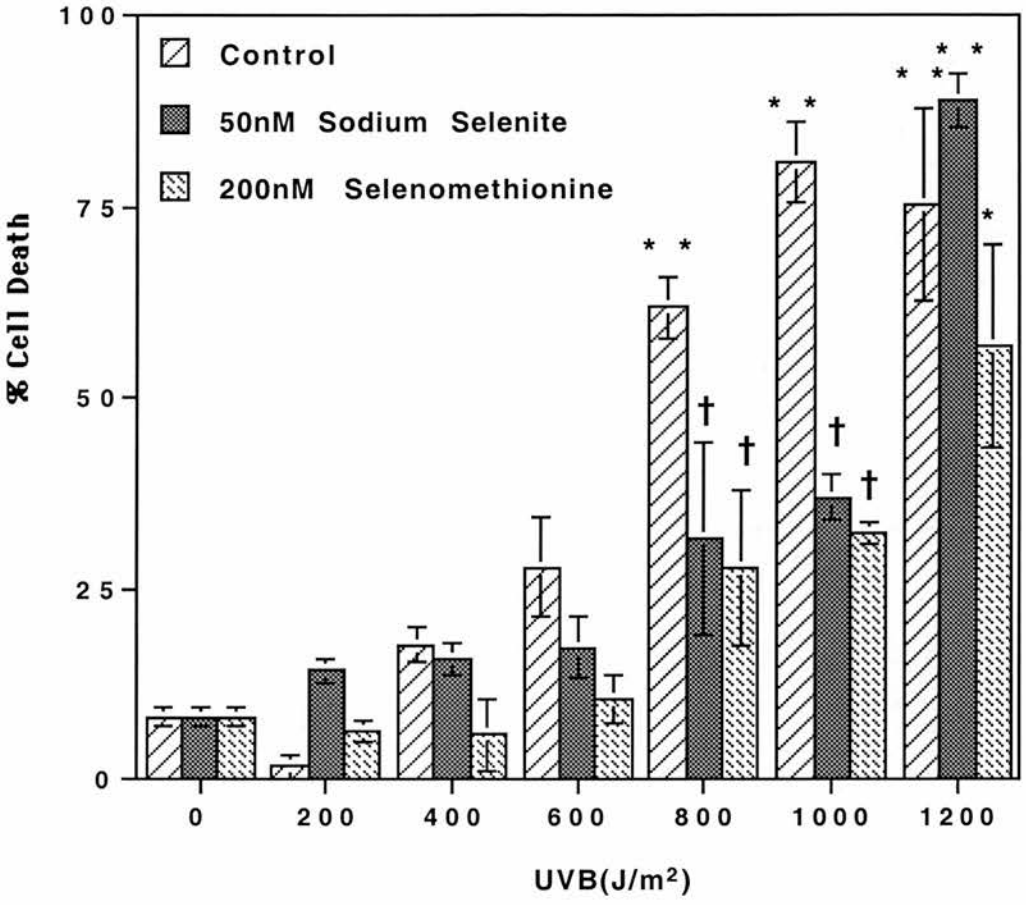
The levels of MDA present were found to decrease slightly immediately following exposure to UVB (Fig 3.15). However 3 hours after exposure to UVB the levels begin to increase slowly over the next 12 hours. Between 12 and 24 hours after exposure to UVB the levels of MDA increased sharply.

To investigate the influence of Se on the formation of MDA following exposure to UVB, HaCaT cells were grown in petri dishes until they were 70% confluent, the cells were then treated with either sodium selenite or selenomethionine for 24 hours. The media was then replaced with PBS and the cells were exposed to 1000 J/m^2 UVB. The original Se-containing media was returned to the cells and they were incubated 24 hours following exposure to UVB. The cells were then harvested and the levels of MDA measured.

Selenium was found to decreases the formation of MDA (Fig 3.16). Sodium selenite decreased the formation of MDA at concentrations in the range of 1-

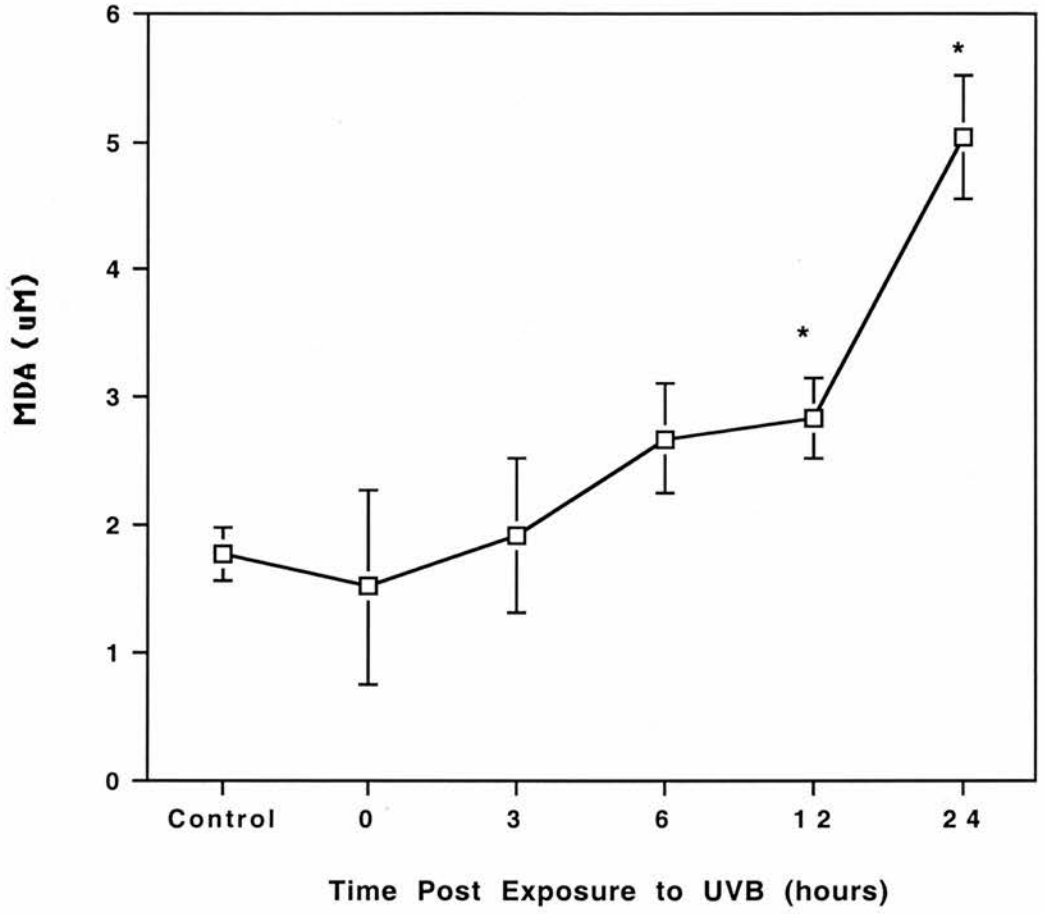
50 nM, and selenomethionine at concentrations between 50-200 nM. HaCaT cells were used in the experiments measuring MDA because a large of volume of cells (at least $1x10^7$) were required to obtain levels of MDA which were detectable. Due to the amount of cells required for each treatment a limited number of concentrations of Se were tested. However the experiments were reproducible and were carried with duplicate samples, twice and with triplicate samples once with similar results.

Figure 3.14: The effect of sodium selenite or selenomethionine added to primary human keratinocytes prior to exposure to various doses of UVB irradiation.



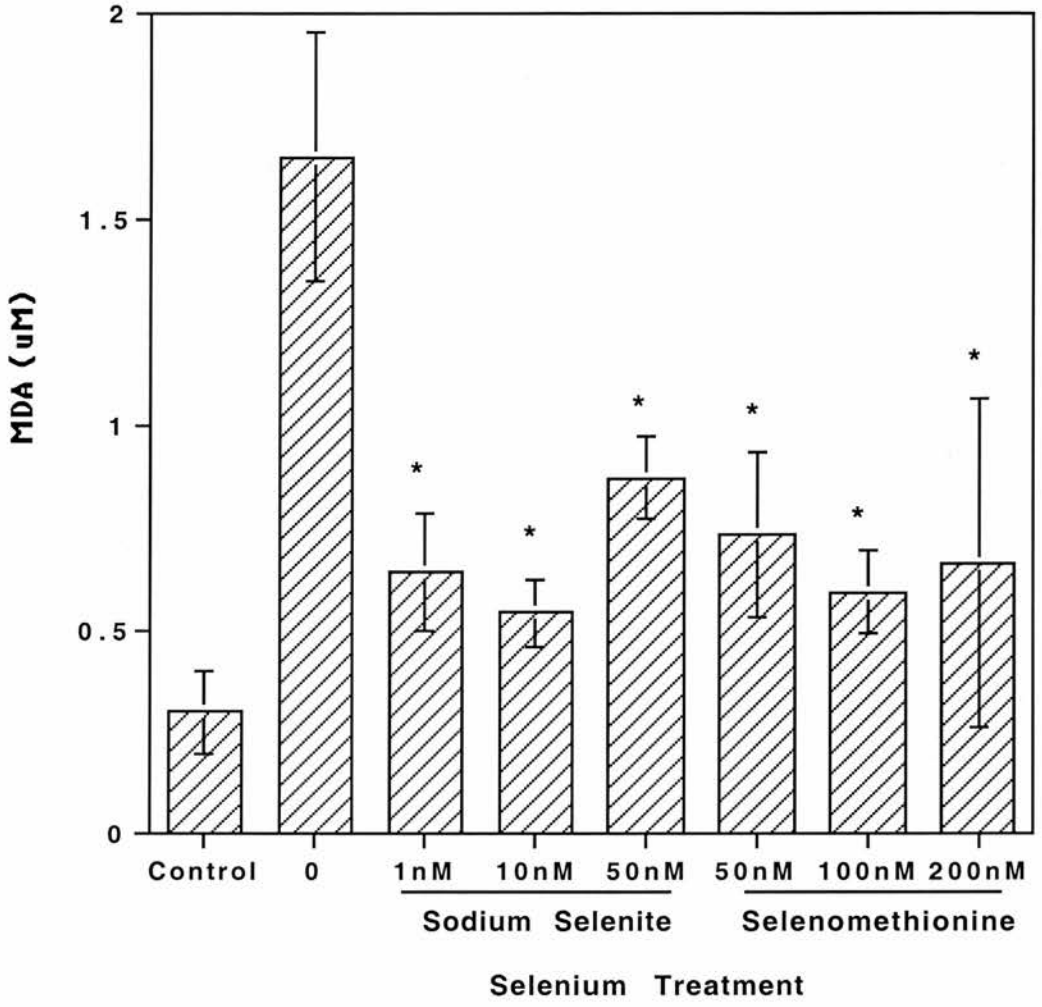
Sodium selenite or selenomethionine was added to the media of primary keratinocytes 24 hours prior to the media being replaced with PBS, and the cells exposed to a range of doses of UVB (200-1200 J/m^2). The original Se-containing media was returned, and the cells were incubated for 48 hours, before cell counts were performed using a haemocytometer and trypan blue exclusion. Control cells received no Se supplement. At least 100 cells were counted. Results are the means of % cell death ± S.E.M, n=3. Significant differences from unirradiated cells, * = $P<0.05$, ** = $P<0.01$. † = Significant protection by Se compared to irradiated control cells.

Figure 3.15: The Effect of UVB at inducing MDA in HaCaT cells.



HaCaT cells were grown to 70% confluence, the media was replaced with PBS and the cells exposed to UVB (1000 J/m^2). The original media was replaced and the cells were incubated for 0-24 hours. The cells were then harvested and the levels of MDA present measure by commercial assay. Control cells were not exposed to UVB. The results are the means of the levels of MDA present ± S.E.M, n=2. Significant increase in MDA compared to control cells, * = $P<0.05$.

Figure 3.16: The Effect of Se on the UVB-induction of MDA in HaCaT cells.



HaCaT cells were grown to 70% confluence, sodium selenite or selenomethionine was added to the media and the cells incubated for 24 hours. The media was replaced with PBS and the cells exposed to UVB (1000 J/m^2). The original Se containing-media was replaced and the cells were incubated for 24 hours. The cells were then harvested and the levels of MDA present measure by commercial assay. Control cells were not exposed to UVB and Se. The results are the means of the levels of MDA present ± S.E.M, n=2. Significant decrease in MDA compared to irradiated cells not treated with Se, * = $P<0.05$.

3.3 Discussion.

3.3.1 Toxicity of Se compounds.

Selenite was considerably more toxic to cells than selenomethionine. Thus treatment with 1 μM selenite, killed approximately 25% of primary keratinocytes and at a concentration of 10 μM, more than 50% of the cells were destroyed. In comparison selenomethionine showed no toxicity at any of the concentrations used in the study (Fig 3.1b). Indeed selenomethionine had to be added at 1 mM before any toxic effect could be observed (Fig 3.10a). Melanocytes were more resistant to the harmful effects of sodium selenite, with toxicity observed at concentrations of 10 μM (44% cell death). As with the keratinocyte study, selenomethionine was not found to be toxic with concentrations as high as 20 μM (Table 3.1b and Fig 3.10b). HaCaT cells were susceptible to cell damage by sodium selenite, with a significant effect on cell viability being found at concentrations of 200 nM. Selenomethionine also appeared to be toxic in HaCaT cells at concentrations of 10 μM (Table 3.2b). The increased toxicity of the seleno-compounds suggests an increased susceptibility to toxic insult in the HaCaT cell line, which may be due to deficiencies in a range of antioxidant enzymes, as a consequence of the HaCaT cells being a spontaneously transformed cell line. Primary fibroblast cells were also found to be susceptible to cell destruction after treatment with sodium selenite at 200 nM but the cells proved resistant to selenomethionine up to 10 μM (Fig 3.30). The results demonstrate a clear difference in how various cells react to treatment with sodium selenite and selenomethionine. In summary, primary keratinocytes were the least susceptible to the harmful effects of sodium selenite, followed by melanocytes, HaCaT cells and finally the most sensitive, fibroblasts. When using selenomethionine all of the cells were unaffected by treatment with concentrations of 10 μM, except the HaCaT cells. The data also show that sodium selenite was more toxic than selenomethionine.

3.3.2 Protective effect of Se on skin cells exposed to UVB.

The results presented in this chapter show that pre-incubation of primary cultures of keratinocytes, melanocytes, fibroblasts and the HaCaT cell line

with Se for 24 hours can provide all cell types with significant protection from UVB-induced cell death (Fig 3.3a, Table 3.4 and Fig 3.4). The chemical form of Se added to cells was an important factor in determining the minimal and optimal concentrations of Se, required to provide protection from UVB. Selenite was more potent than selenomethionine at conferring protection, illustrated by the finding that in melanocytes, keratinocytes and HaCaT cells, significant protection was achieved with a concentration of selenite as low as 1 nM, whilst no significant protection was achieved with selenomethionine below concentrations of 10 nM. Concentrations of Se which conferred maximal protection to cells also varied with the chemical form of Se. With selenite, maximal protection was achieved using a concentration of 10-50 nM in all cell types. However when using selenomethionine, maximal protection was achieved at concentrations of 50 nM, for keratinocytes, and 100 nM for HaCaT's, fibroblasts and melanocytes (Fig 3.3a, Table 3.4 and Fig 3.4). The observation that a similar degree of protection from UVB-induced cell death by Se addition was found with the HaCaT keratinocyte cell line and primary keratinocytes, suggests that the phenomenon may be a general one amongst keratinocyte cell types.

There are some problems when using the trypan blue exclusion assay to assess cell death as it has been demonstrated that apoptotic cells can exclude the dye, however the effect of Se on apoptotic cell death has been investigated in detail in Chapter 4. Also the trypan blue exclusion method can give an overestimate of cell survival as cells may exclude the dye but may not be able to replicate. To avoid this problem some of the key experiments with primary keratinocytes were repeated with the colony formation assay. It was demonstrated in keratinocytes that the results from the trypan blue assay, compared favourably with the results obtained using the colony formation assay. It should be noted however that with the colony formation assay a much lower level of UVB exposure must be used, as the levels of UVB required to kill cells are far greater than the doses required to prevent cell replication. In the keratinocytes 960 J/m^2 was used in the trypan blue experiments and only 200 J/m^2 in the colony formation assay Fig 3.3a and 3.3b).

Selenium-induced protection from UVB-induced cell death has been reported by several other groups. The majority of studies have been

performed using human skin fibroblast supplemented with sodium selenite. Protection from cell death by UVB radiation (Richard *et al*, 1990), UVA radiation (Leccia *et al*, 1993; Moysan *et al*, 1995) and a decrease in the number of sunburn cells in human skin have been demonstrated (La Ruche and Cesarini, 1991). The concentrations of selenite used in the experiments in the literature were very high and were non-physiological, with 1.2 μM sodium selenite used in a study by Richard *et al* and 1.3 μM used in another study by Leccia *et al*,. Concentrations of sodium selenite over 1 μM produced toxic effects in fibroblasts in the experiments presented in this thesis. However, experiments carried out by Moysan *et al* using fibroblasts and exposure to UVA to induce cell death, showed significant protection with selenite at concentrations of 320 nM.

It is widely held that Se exerts many of its biochemical actions through the expression of specific selenoproteins, in which Se is inserted at specific selenocysteine residues encoded by a TGA triplet. Insertion of Se at these TGA-directed sites, requires Se to be presented in a chemically active form similar to selenide and current evidence indicates that selenite, is a more potent precursor of selenide than selenomethionine (Sunde, 1990). However, although whole body turnover studies in man have shown that selenomethionine is re-utilised by cells more efficiently than inorganic forms, this appears to arise because selenomethionine can be substituted into proteins non-specifically for methionine. When replacing methionine residues in proteins, selenomethionine appears to have no bioactivity (Waschulewski and Sunde, 1988).

3.3.3 Possible mechanisms of Se protection from UVB-induced cell death.

We have shown that pre-incubation with Se can prevent UVB-induced damage, the mechanism by which this occurs is not clear. Se did not exert a protective effect through altering cell growth patterns at the concentrations which were protective (Fig 3.1a, Table 3.1a, Table 3.2a, and Table 3.3). In order to demonstrate that the Se compounds were not acting as sunscreens on the cells, in all of the experiments the cells were washed well and irradiated in PBS with no Se present (Fig 3.7). However, this did not exclude the possibility that the cells had free intracellular Se present, which could absorb the UVB. However when the absorption spectra of the concentrated

Se solutions made in PBS were measured, it was found that neither Se compound absorbed in the UVA spectrum and selenomethionine only had a small level of absorption in the UVB spectrum. The lack of absorption suggests that cells were not being shielded from UV radiation due to the Se compounds acting as a sunscreen.

It has been clearly shown that cells need to be pre-incubated with Se before any protection from UVB-induced damage is achieved (Fig 3.4). As when Se was added immediately after UVB exposure little protection was achieved (Fig 3.6).

Overall the data suggests that Se may be acting through incorporation into selenoproteins, rather than by a direct anti-oxidant chemical action. Selenomethionine had to be present on the cells for at least 24 hours to obtain significant protection and 12 hours pre-treatment was required for sodium selenite (Fig 3.8). The extended period of time required for pre-incubation with seleno-compounds is probably essential to allow the synthesis of selenoproteins. Sodium selenite was observed to be more rapid in having an effect than selenomethionine, probably because selenite has been demonstrated to be a more suitable precursor to selenide, itself the precursor of selenocysteine (Sunde, 1990). Also as demonstrated in Fig 1.3 in Chapter 1, selenite requires fewer steps to be converted into selenide than selenomethionine (Foster and Sumar, 1997). Furthermore, selenomethionine can be substituted into proteins non-specifically for methionine. The protective effect Se had against UVB-induced damage increased further with a 48 hour pre-incubation period with Se.

In an attempt to confirm that selenoprotein synthesis is required for protection, cycloheximide was added to the cells with the Se. However the data showed that cycloheximide alone had toxic effects on the cells following exposure to UVB. The toxic effect of cycloheximide was possibly due to the cycloheximide inhibiting the production of essential heat shock proteins, which help the cell respond to toxic insults. Heat shock proteins have been detected in human skin (Trautinger *et al*, 1993) and are associated with the regulation of the cell proliferation, differentiation, the inhibition of sunburn cell formation and UV-induced cell death (Trautinger *et al*, 1995; Kane and Maytin, 1995; Maytin *et al*, 1994). Thus although the protective effect of Se

was prevented by cycloheximide, this cannot be regarded as evidence for Se acting through selenoprotein synthesis.

If Se needs to be incorporated into selenoproteins for it to exert protection against UVB, possible candidate antioxidant selenoproteins include, the families of GPXs and TRs.

Labelling of tissues with [^{75}Se]selenite has demonstrated the presence of more than 30 selenoproteins and the pattern of selenoprotein expression differs between tissues. It is thought that some of the uncharacterised selenoproteins may be involved in detoxification reactions and in modifying cell growth or cell death (Sunde, 1990). In Chapter 8, the effect of Se on the expression of the GPX and TR proteins will be discussed in more detail.

Proteins of the GPX family are obvious potential candidates for selenoproteins which may mediate the protective effects of Se, as these enzymes are capable of detoxifying hydrogen peroxide, lipid hydroperoxides and phospholipid hydroperoxides which are produced during UVB exposure. GPX activity is resistant to UV irradiation unlike SOD and CAT activity which both dramatically fall following irradiation (Pence and Naylor, 1990; Shindo *et al*, 1994).

Shisler *et al* demonstrated that if a GPX-like gene is transfected into HeLa and HaCaT cells, the cells become resist to UVB-induced cell death. The induction of cell death in HeLa cells was decreased from 60% to 10%, and in HaCaT cells from 75% to 20% (Shisler *et al*, 1998). The reduction in cell death indicates that GPX has a crucial role in protecting the skin from UVB-induced damage and that this is due to the antioxidant effects of GPX. The level of protection reported by Shisler *et al* is similar to that found in the present study. In keratinocytes, melanocytes and HaCaT cells the level of cell death was decreased from 70-80% to 15-25% after Se pretreatment (Fig 3.3a, Table 3.4). The protective actions of the GPX-like protein suggest a role for GPX in protecting the skin from UVB-induced damage. However the protection may also be provided by other selenoproteins

TR is a selenoenzyme which has multiple functions, it can reduce oxidised thioredoxin (Holmgren and Bjornstedt, 1995) and can directly reduce

hydrogen peroxide, lipid hydroperoxides and superoxide in the presence of NADPH (Bjornstedt *et al*, 1995a). Unlike other antioxidant enzymes TR is in a unique position, as it is located in the cytosol and on the plasma membrane of skin cells (Schallreuter *et al*, 1986a; Schallreuter and Wood, 1986). Schallreuter and Wood have also reported that TR plays an essential role in the pigmentation of the skin following exposure to UVB, with lighter skin types containing lower levels of TR in the skin (Schallreuter and Wood, 1989) Thus TR plays an important role in the reaction of the skin to UV and in protecting the skin from UVB-induced damage.

Lipid peroxidation is a form of oxidative damage caused by exposure to UVB. Therefore it can act as an indicator of whether Se is decreasing oxidative stress, produced by exposure to UVB in the skin. Numerous antioxidants can decrease the formation of lipid peroxides in the skin following exposure to UV. The antioxidants which can decrease lipid peroxidation include; topically applied glutathione, which acts to decrease the formation of TBARS in irradiated mouse skin (Kobayashi *et al*, 1996b). Ascorbic acid injected intraperitoneally or intracutaneously decreases the formation of TBARS in mouse skin following exposure to UVB (Kobayashi *et al*, 1996a). Finally vitamin E added immediately after exposure to UVB is reported to decrease the formation of TBARS in human skin fibroblasts by at least 50% (Kondo *et al*, 1990).

Selenium has also been found to decrease the formation of TBARS in patients. Patients given Se and then exposed to a solar simulator displayed decreased levels of TBARS (Pietschmann *et al*, 1992). Furthermore, cultured fibroblasts treated with sodium selenite (320 nM) prior to exposure to UVA, exhibited a 50% decrease in the formation of TBARS (Moysan *et al*, 1995; Leccia *et al*, 1993). Similar results were found in my study with, Se treatment diminishing the formation of MDA in HaCaT cells following exposure to UVB. The decrease in MDA formation indicated that Se is indeed decreasing the oxidative stress induced by UVB. Any decrease in oxidative stress would increase the cells ability to survive exposure to UVB. As already alluded to, one mechanism by which Se could decrease the level of oxidative stress is by the increasing the expression of antioxidant selenoproteins.

3.3.4 Loss of protection to UVB at high concentrations of Se.

Although both Se compounds displayed concentration-dependent protection from UVB damage up to approximately 100 nM, when the concentration of Se was further increased to 1 μM protection appeared to diminish in all cell types. For selenite, this loss in protection with increasing concentration of selenite is at least in part due to a direct toxic effect of selenite and its metabolite selenodiglutathione when present at high concentrations (Stewart *et al*, 1999). Selenite has a directly toxic action on cells and a recent study has shown that the metabolite selenodiglutathione is also a potent cytotoxic agent (Wu *et al*, 1995). For selenomethionine the mechanism behind the loss of protection at higher concentrations is unclear, since selenomethionine was not toxic at any of the concentrations used (Fig 3.1b, Table 3.1b, Table 3.2b and Table 3.3). It is possible that UVB exposure might modify selenomethionine to produce a toxic metabolite, but I could find no evidence of increased toxicity in solutions of selenomethionine or sodium selenite irradiated with UVB and incubated on keratinocytes (Fig 3.11). Alternatively, 1 μM selenomethionine although not toxic on its own, may be close to a concentration of the compound that was noxious to the cells. When combined with UVB exposure the two agents may act in concert to provoke cell death. However, when cells were exposed to higher concentrations of selenomethionine it was demonstrated that selenomethionine was only toxic to melanocytes at concentrations of 50 μM (Fig 3.10b) and to keratinocytes at 1 mM (Fig 3.10a). Concentrations of over 50 μM are much higher than any employed in this study so do not explain the loss of protection in keratinocytes or melanocytes measured. The loss of protection seen when using high concentrations of selenomethionine in HaCaT cells, can be explained, by the fact that it becomes toxic to the cells at 10 μM.

Another possibility is a recent observation in our laboratory using HaCaT cells, that with increasing concentrations of Se, GPX activity increases, but at concentrations above 200 nM the GPX activity declines (M Lewin, private communication). Furthermore TR activity is also inhibited by high concentrations of selenite (Bjornstedt *et al*, 1995b). If the decreases in activity of GPX and TR are caused by increasing the concentration of Se, then this could explain the loss of the protective effect observed in this study.

3.3.5 Effect of Se at preventing other forms of oxidative damage.

In order to demonstrate if the loss in protection seen at higher concentrations of selenomethionine was common to other forms of oxidant insult, menadione toxicity towards keratinocytes was studied. The experiments with menadione also provided information on whether Se protected against other oxidative assaults. Menadione produces free radical-induced damage including; the production of superoxide, hydrogen peroxide, lipid peroxides and oxidative damage to DNA (Tzeng *et al*, 1995; Woods *et al*, 1997). Cells were supplemented with Se for 24 hours, and subsequently pulsed with menadione for 3 hours. As shown in Fig 3.13 it was discovered that sodium selenite protected keratinocytes from menadione induced cell death (Fig 3.13). Protection was observed with the same concentration range of selenite (10-100 nM) as was found for UVB-induced cell death (1-200 nM)(Fig 3.3a). Selenomethionine however did not offer the cells substantial protection from menadione toxicity, only proving effective at 50 nM. The difference in protection against menadione elicited by the seleno-compounds highlights again, the contrasting abilities and properties they exhibit. The toxic mechanisms of menadione may differ from that of UVB, this may explain why selenomethionine was not as protective against menadione induced cell death. Also it can be seen in Fig 3.13 that selenomethionine does indeed lose it protective effect at high concentrations.

3.3.6 Protection by antioxidants against UVB-induced cell death.

Other antioxidants have also been shown to protect human keratinocytes from UVB-induced cell death and apoptosis e.g. α-tocopherol decreases UVB-induced cell death (Malorni *et al*, 1996; Kondo *et al*, 1990) and ascorbic acid has also been demonstrated to decrease UVB-induced cell death in HaCaT cells (Savini *et al*, 1998). These studies serve to reinforce the hypothesis that, UVB exposure produces free radical species in the skin and that antioxidants such as selenoproteins can protect the skin cells by inactivating these reactive molecules.

3.3.7 Susceptibility of skin cells to UVB-induced cell death.

It was found that keratinocytes and HaCaT cells were more resistant to UVB-induced cell death than melanocytes; typically a UVB dose of 720 J/m^2 was required to produce 75% cell death in melanocytes (Table 3.4) whilst 960 J/m^2 was required to produce 75-80% cell death in keratinocytes and HaCaT cells (Fig 3.3a and Table 3.4). Fibroblasts appeared to be more susceptible to cell destruction by UVB radiation than other skin cells. However, as already described, this was measured by the more sensitive colony formation assay and so cannot be directly compared to the trypan blue experiments. However, on the colony formation assay 150 J/m^2 was used to decrease colony formation for the fibroblasts and 200 J/m^2 for the keratinocytes.

3.3.8 Finite levels of antioxidants in the skin.

When the protective effect of Se was studied at higher doses of UVB the protective effect began to decrease, with increasing UVB dose. This decrease in protection has been reported before by other groups. Selenium supplementation can protect fibroblasts and endothelial cells from H_2O_2-induced cell death (50 nM sodium selenite), however at higher levels of H_2O_2 the protection is lost (Leist *et al*, 1996). At increasing levels of damage the cell's antioxidant systems appear to become overwhelmed and any protective effect is lost. The loss of cellular defence against oxidative insult is a consequence of the limited capacity of the intracellular antioxidant system. When confronted with high levels of free radicals a cell can provide a limited response with its antioxidants, before requiring a recovery period.

3.4 Summary.

I have shown that Se can protect keratinocytes, fibroblasts and melanocytes from UVB-induced damage and such protection can be achieved using very low concentrations of Se, particularly when presented as sodium selenite. The protective effect is not due the Se increasing cell growth or acting as a sunscreen. Cells need to be pre-incubated with the Se compounds for at least 24 hours before significant protection is achieved, suggesting that the Se exerts its effects through synthesis of antioxidant selenoproteins. One mechanism by which Se may be protecting the cells is by decreasing the level

of oxidative stress induced by exposure to UVB, presumably by synthesis of antioxidant enzymes such as TR and GPX. The decrease in oxidative stress was indicated by a decrease in the formation of lipid peroxides

Our data in isolated cells would suggest that, as in the mouse, (Pence *et al*, 1994), increasing Se intake could afford protection from the harmful effects of UVB radiation. Furthermore, our results suggest that if supplements of Se were to be given, inorganic forms such as selenite may be more potent at providing this protection than organic forms such as selenomethionine. However inorganic forms such as selenite are also more toxic than organic forms.

3.5 Future work.

Specific TR and GPX inhibitors could be employed, to assess which enzyme system plays an important role in protecting the cells from UVB-induced cell death. Gold-thioglucose could be utilised as it inhibits selenoproteins, however this may not prove to be specific. Studies into the levels of selenoproteins following Se addition might give an indication of which selenoproteins are important at protecting the cells (Chapter 8). Cells could also be made to overexpress TR and GPX and their response to UV could be measured.

Chapter 4

Effect Of Se On The Level Of Apoptosis In Primary Human Keratinocytes Exposed To UVB.

4.1 Introduction.

It is currently understood that there are two mechanisms involved in cell death, necrosis and apoptosis. Necrosis is caused by an episode of acute and severe cellular injury, resulting in the loss of the cell membrane integrity and lysis. Apoptosis in comparison is an active form of cell death with specific morphological features which differ from necrosis (Kerr *et al*, 1972). The mechanisms and pathways of apoptosis, including the role of p53 are covered in depth in Chapter 1, Section 1.4.1, however some of the main points are discussed briefly in the following sections.

Many specific biochemical changes occur in apoptotic cells which involve the activation of catabolic enzymes. The DNA undergoes intranucleosomal cleavage by endonucleases, which fragment the DNA into approximately 200 base pair multi-mers. This feature of apoptosis can be demonstrated in cultured cells undergoing apoptosis. The DNA is separated by agarose gel electrophoresis and a characteristic series of bands called the nucleosomal ladder can be observed (Wylie *et al*, 1984).

Apoptotic keratinocytes are characterised by "a condensed, basophilic nucleus and eosinophilic homogenisation of the cytoplasm" (Young, 1987). Keratinocytes undergoing apoptosis are often referred to as being dyskeratotic cells or sunburn cells (Young, 1987). In culture, apoptotic keratinocytes also lose their cell to cell contacts, round up and develop cell surface blebs before detaching from the cell monolayer (Malorni *et al*, 1994).

When induced, apoptosis is mediated by two intracellular pathways, the Fas/FasL pathway (which is related to the TNF/TNFR pathway) and the p53 tumour supressor pathway. The discrete pathways are described in detail in Chapter 1, Section 1.4.1. Keratinocytes can express Fas, FasL, TNFR,

TNF and p53, therefore apoptosis can be mediated by the Fas or the p53 pathway.

Exposure to UV stimulates both Fas and FasL expression on keratinocytes and antibodies to anti-FasL, partially block the induction of UV-induced apoptosis in keratinocytes (Leverkus *et al*, 1997). Exposure to UV also causes Fas molecules to cross link on the surface of cells and this can activate apoptosis (Aragane *et al*, 1998). This process is thought to involve superoxide anions and hydroxyl radicals (Gorman *et al*, 1997). Exposure to UV induces expression of TNFR and TNF-α production. Moreover, antibodies to TNF-α partially block UVB-induced apoptosis in keratinocytes (Schwartz *et al*, 1995).

The tumour supressor protein p53 is post-translationally stabilised by UV radiation, prior to the induction of apoptosis in keratinocytes (Henseleit *et al*, 1997). The level of apoptosis in the epidermis following exposure to UV radiation, is greatly decreased in p53 knockout mice (Ziegler *et al*, 1994). Exposure to UVB causes DNA damage which leads to the induction of p53 (Lu and Lane, 1993). If the amount of DNA damage is limited, then the cell undergoes G1 cell cycle arrest and the damaged DNA is repaired before the cell is allowed to progress through the cell cycle (Lane, 1992; Cox and Lane, 1995; Cox *et al*, 1995). If the damage is severe then the cell will undergo apoptosis, which will prevent the mutated DNA from being passed on to daughter cells.

In damaged or stimulated cells the cellular level of p53 protein increases due to protein stabilisation and this rise is not prevented by RNA or protein synthesis inhibitors (Maltzman and Czyzyk, 1984). The p53 protein has to be phosphorylated to become stable and then phosphorylated again to bind DNA, these events are covered in more detail in Chapter 1, section 1.4.1. Therefore, the abundance of p53 protein does not correlate with p53 activity, as the p53 protein has to be activated to bind DNA tightly.

Some of the genes which p53 regulates are $p21^{WAF1/CIP1}$ (EL-Deiry *et al*, 1994) and GADD45 (Zhan *et al*, 1994), which are involved in the p53 response to DNA damage and cell cycle arrest. The method of action of $p21^{WAF1/CIP1}$ and GADD45 are described in Chapter 1, Section 4.3. It has

been shown that p53 can down regulate Bcl-2 expression and increase Bax expression, which can lead to the induction of apoptosis (Miyashita *et al*, 1994, reviewed in Hoffman and Liebermann, 1994).

In many tumours including those of the skin, the regulators of apoptosis often do not function correctly. Apoptosis inhibitors such as Bcl-2 are increased, or p53 may be inactivated by mutations. In more than 50% of human malignancies the gene for p53 is altered, a fact that underlines the importance of p53 in controlling normal cell growth (Hollstein *et al*, 1991; reviewed in Raff, 1998).

Reactive molecules such as H_2O_2, which induce oxidative stress can stimulate cells to undergo apoptosis (Lennon *et al*, 1991). In this context, it has been shown that TNF-mediated apoptosis can be inhibited in many cell types by antioxidants such as thioredoxin (Matsuda *et al*, 1991) or N-acetylcysteine (Chang *et al*, 1992). Furthermore, Fas-induced apoptosis is reduced by the addition of thioredoxin and SOD (Matsuda *et al*, 1991; Hirose *et al*, 1993). Reactive oxygen species-mediated DNA damage has been demonstrated to lead to the accumulation of p53 (reviewed in Buttke and Sandstrom, 1994). The antioxidant N-acetylcysteine has been shown to inhibit the UV activation of p53, suggesting an involvement of oxidative stress in the activation of p53 (Renzing *et al*, 1996).

Cellular symptoms of apoptosis such as membrane blebbing in keratinocytes can be decreased by the addition of antioxidants such as α-tocopherol (Malorni *et al*, 1996), glutathione (Godar, 1999), SOD and catalase (Miyachi *et al*, 1983).

It has been observed that p53 can activate the antioxidant selenoprotein GPX, indeed there is a p53 binding site within the promoter for GPX. This may be a protective mechanism to reduce oxidative damage to the cell while p53 halts the cell cycle to repair DNA damage. The biological importance of p53's activation of GPX is not fully understood as yet (Tan *et al*, 1999).

The aims of this study were to -:

- Investigate the potential induction of apoptosis by UVB in primary human keratinocytes.

- Study the effects of Se on the potential induction of apoptosis by UVB.

- Investigate the effect exposure to UVB has on the levels of p53 protein.

- Study the impact of Se on the abundance of p53 protein after exposure to UVB.

4.2 Methods and Results.

4.2.1 Induction of apoptosis by UVB in primary human keratinocytes.

4.2.1.1 Quantitated by acridine orange staining.

Apoptosis was quantitated using acridine orange staining. Primary human keratinocytes were grown to 70% confluence in six well dishes, the media was replaced with PBS and the cells exposed to 480, 720 or 960 J/m^2 UVB. The original media was replaced and the cells were left for 6, 16 or 24 hours, prior to being stained with acridine orange and counted using fluorescent microscopy (Chapter 2, section 2.4.1). The percentage of apoptotic cells was calculated. Each time point consisted of six replicates. Apoptotic cells show apoptotic body formation and stain very brightly with acridine orange (Fig4.1b)

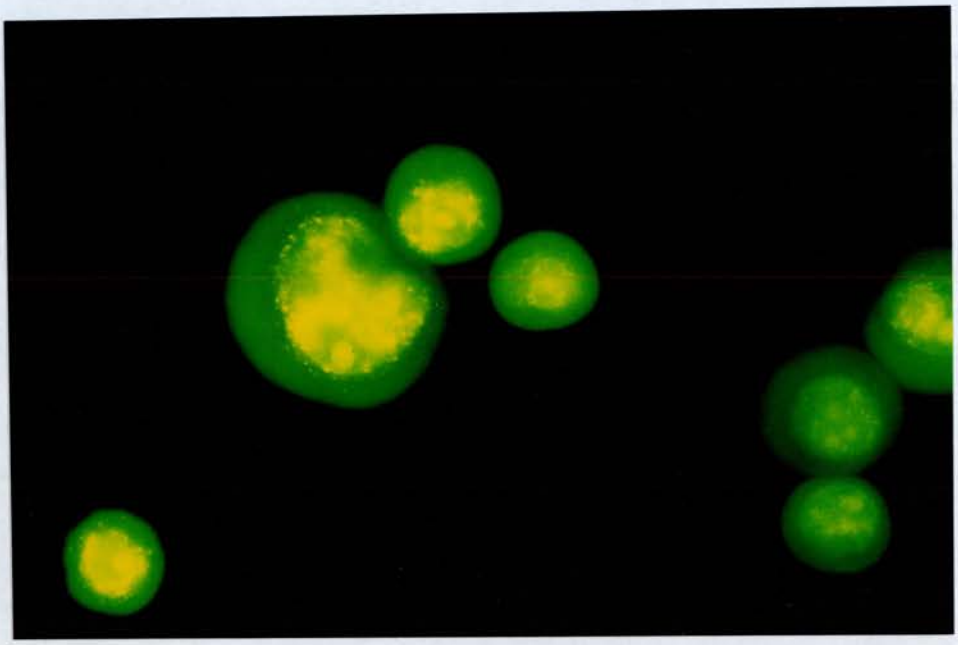
Normal cells stained with acridine orange and viewed using a fluorescent microscope, appear large, circular and green. The nuclei are bright green/yellow as the dye binds to the DNA, the nuclei also appear large and diffuse (Fig 4.1a). Apoptotic cells appear to be much brighter than normal cells and have a shrunken appearance. They also have apoptotic bodies budding off from the cell, which are bright yellow and the nucleus can no longer be seen (Fig 4.1b).

The amount of apoptosis induced was dependent on both the dose of UVB and the time following exposure to UVB (Fig 4.2). The lowest dose of UVB (480 J/m^2) had a peak of apoptosis at 24 hours following exposure to UVB. The highest dose of UVB (960 J/m^2) however caused a peak in apoptosis at 6 hours after exposure, whilst 720 J/m^2 caused a peak at 16 hours following exposure. The greatest number of apoptotic cells was found at 720 J/m^2 at 16 hours following exposure to UVB. This time point was selected for the rest of the study, however a slightly lower dose of UVB (600 J/m^2)was selected for the rest of the study, as it was deemed important not to induce necrosis of the cells. *

Continued on page 4-8

Figure 4.1: Acridine orange stained normal and apoptotic keratinocytes.

(a) Unirradiated primary human keratinocytes stained with acridine orange (x200).



(b) Primary human keratinocytes 16 hours after being exposed to 600 J/m^2 UVB (x200).

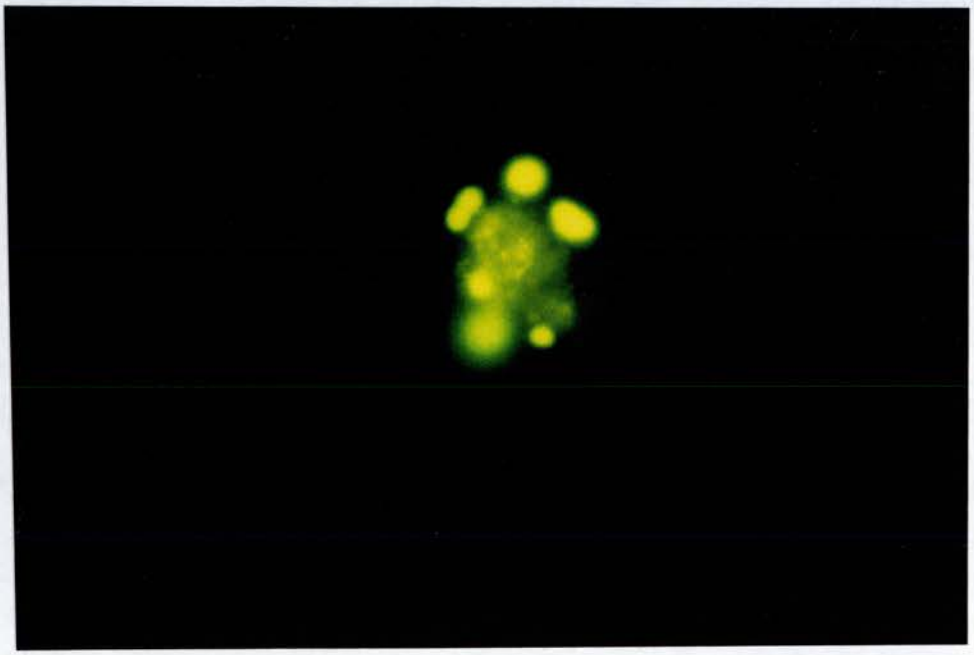
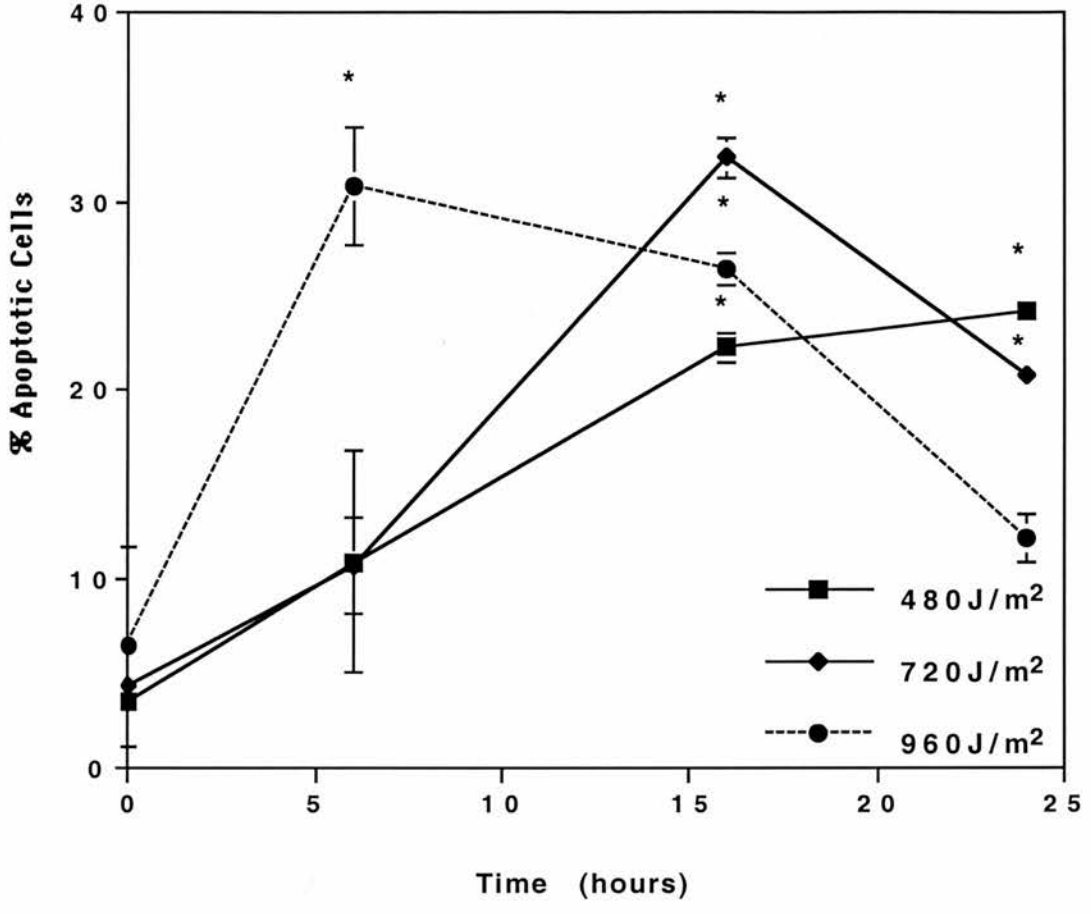


Figure 4.2: Time course for the induction of apoptosis in primary human keratinocytes following exposure to UVB.



Cells were grown to 70% confluence the media was replaced with PBS and the cells exposed to UVB. The original media was replaced and the cells were incubated for 6-24 hours. The cells on the monolayer and supernatant were harvested separately, resuspended in PBS, stained with acridine orange and counted using a fluorescent microscopy. The 0 hour cells were unirradiated. Results are the means of the % apoptosis ± S.E.M, n=6. Significant difference from unirradiated cells, * = $P<0.05$.

4.2.1.2 Confirmation of apoptosis using electron microscopy.

When studying the process of apoptosis it is essential to confirm that the cells are apoptotic by several methods.

Primary keratinocytes were grown to 70% confluence in 10 cm petri dishes, the media was removed and replaced with PBS and the cells were exposed to 600 J/m^2 UVB. The original media was replaced and the cells were incubated for 16 hours, before being processed for electron microscopy (Chapter 2, section 2.4.2). The time point and dose of UVB used were found to be optimal for the induction apoptosis measured using acridine orange staining (Fig 4.2). The cells were then studied using an electron microscope to distinguish any apoptotic features. The irradiated keratinocytes were demonstrated to be undergoing apoptosis and cells in different stages of apoptosis could be demonstrated.

- Normal unirradiated cells have a regular round appearance, with a large diffuse nucleus (Fig 4.3a).

- The cytoplasm of irradiated cells first becomes foamy in appearance and the chromatin begin to condense (Fig 4.3b).

- The nucleus begins to shrink, with the nuclear membrane remaining intact (Fig 4.3c).

- The nucleus continues to shrink and the cell begins to form apoptotic bodies which are membrane bound (Fig 4.3d) and finally the apoptotic bodies, which are still membrane bound detach from the cell (Fig 4.3e)

4.2.2 Effect of Se on UVB-induced apoptosis.

4.2.2.1 Measured by acridine orange staining.

Six well dishes of primary keratinocytes were incubated with either sodium selenite or selenomethionine for 24 hours prior to the media being replaced and the cells exposed to 600 J/m^2 UVB. The original Se-containing media *

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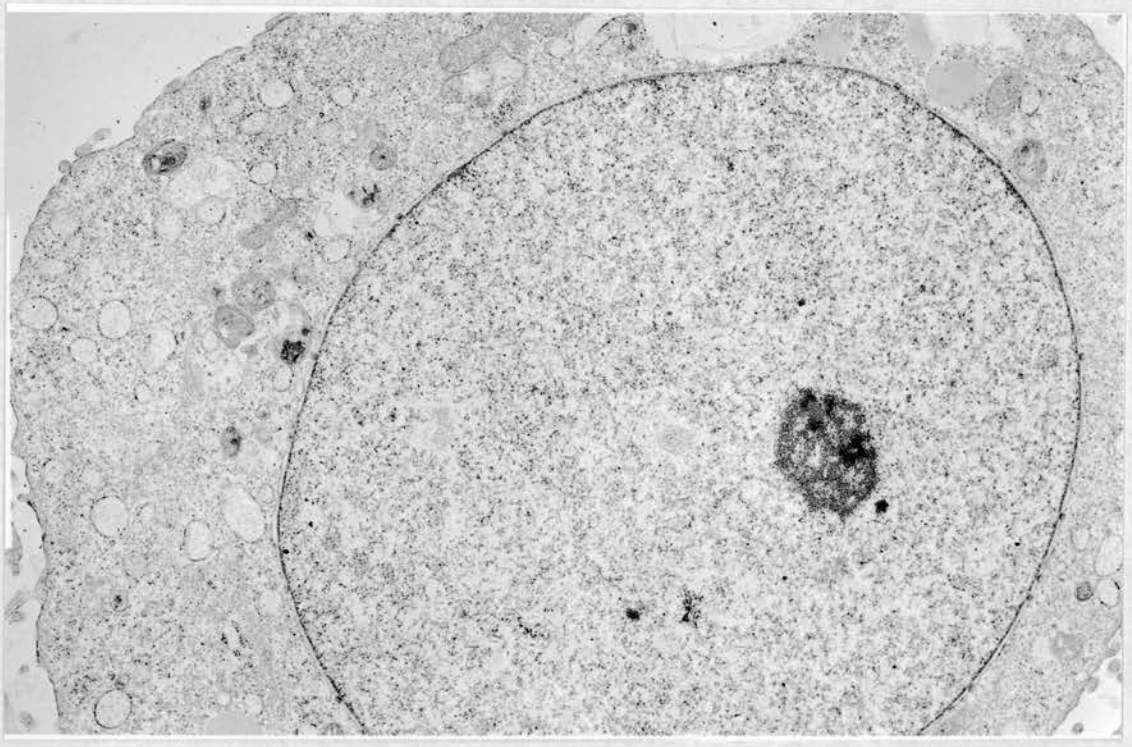
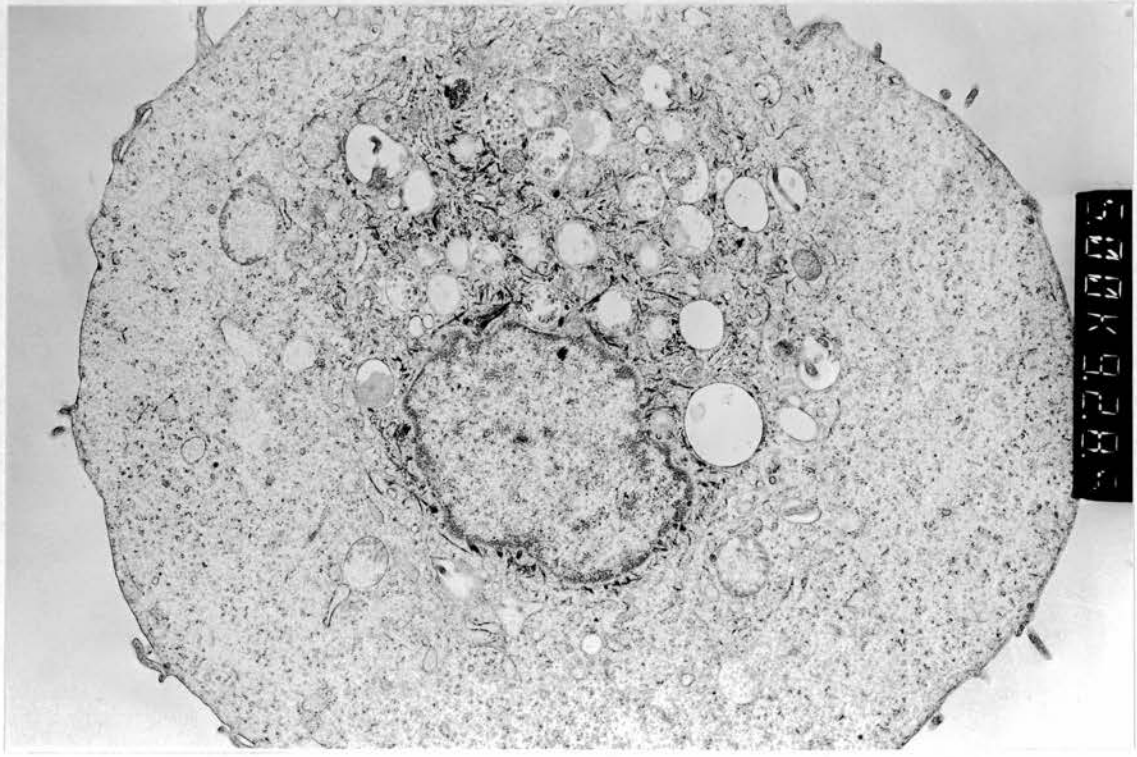
Figure 4.3: Electron microscopy on unirradiated and irradiated keratinocytes.

(a) Unirradiated primary human keratinocytes (x5000).

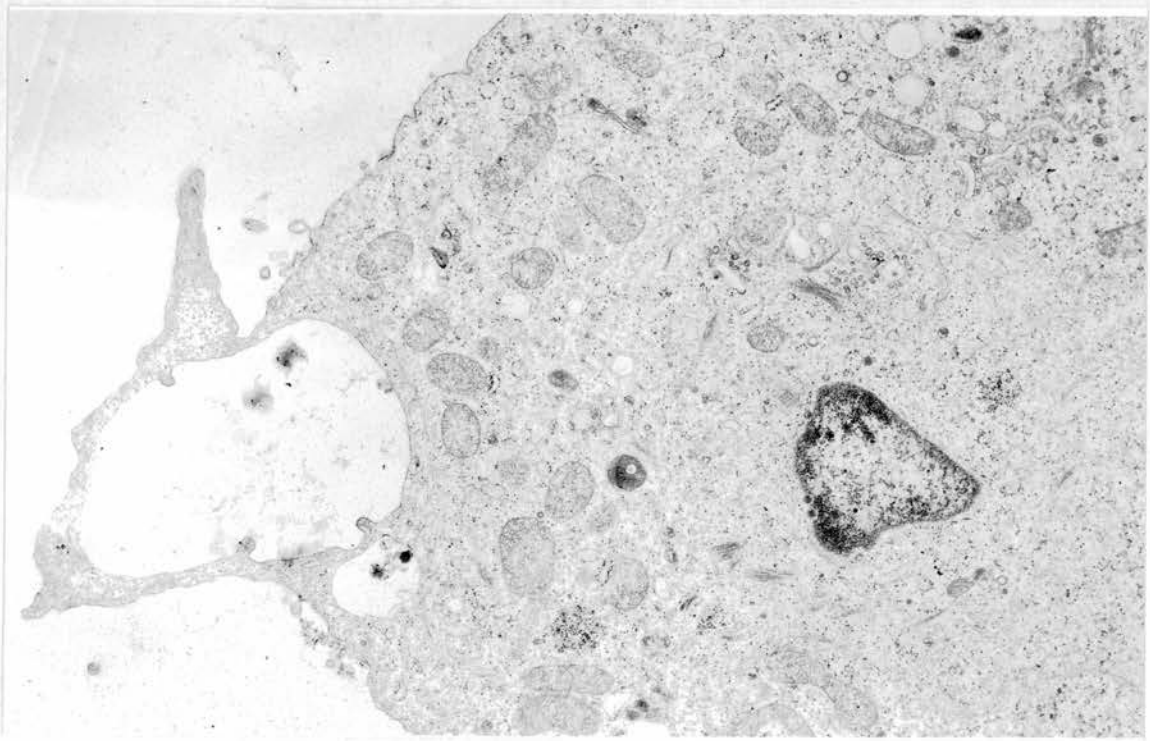


(b) Irradiated keratinocytes 16 hours after exposure to UVB (x5000).-The cytoplasm is foamy, and the chromatin is starting to condense.

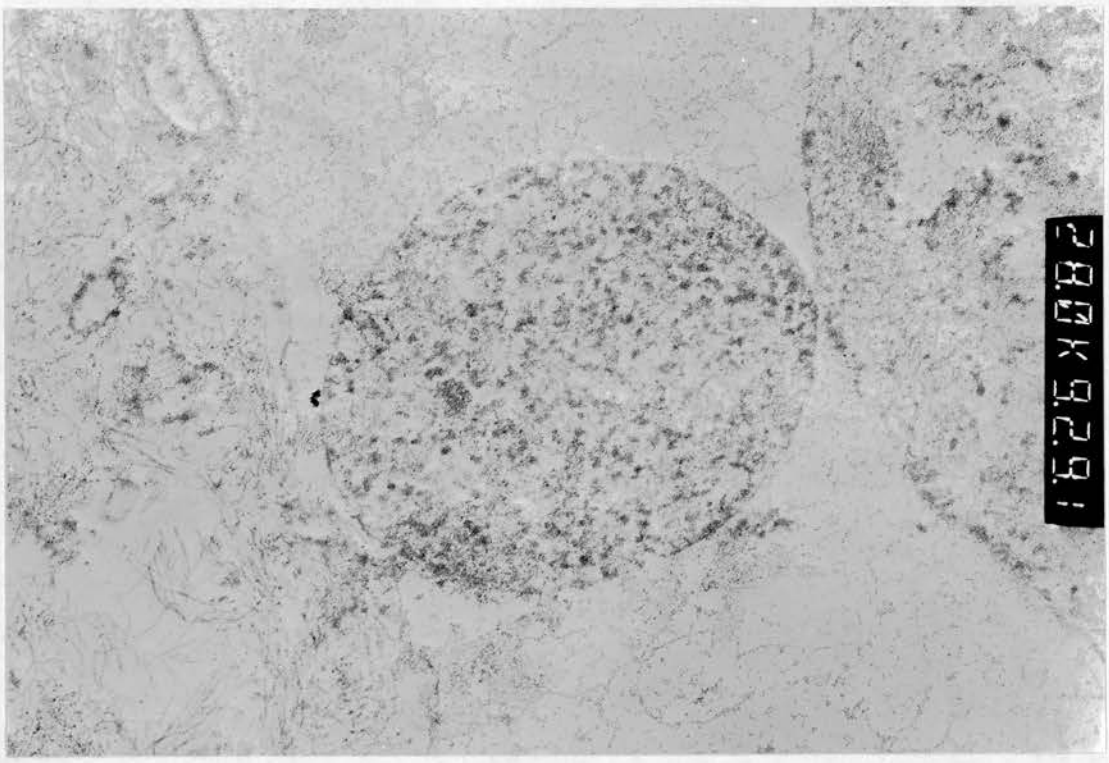


(c) **Irradiated keratinocytes 16 hours after exposure to UVB (x5000).** -The nucleus has begun to shrink.

(d) **Irradiated keratinocytes 16 hours after exposure to UVB (x5000).** -The nucleus is very small and the cell membrane has started to form apoptotic bodies.



(e) Irradiated keratinocytes 16 hours after exposure to UVB (x28,000). - An example of a membrane bound apoptotic body.



Primary keratinocytes were grown in 10 cm^2 petri dishes, the media was replaced with PBS and the cells were exposed to 600 J/m^2 UVB. The media was replaced and the cells incubated for 16 hours. The supernatant and monolayer were harvested separately and dehydrated in alcohol gradients. The cell pellet was then embedded in araldite and thin sections cut for electron microscopy. Unirradiated cells are shown in Fig 4.3a and irradiated cells in Figs 4.3b-e.

was then returned to the cells and the cells incubated for a further 16 hours. The cells were then harvested, stained with acridine orange and the percentage of apoptotic cells was calculated as described previously in Chapter 2, section 2.4.1. The experiments were carried out with a n of 6, three times.

Exposure to UVB induced apoptosis in 30% of cells. Control cells showed a low background level of apoptosis (5%)(Fig 4.4). Selenium decreased the induction of UVB-induced apoptosis in a dose-dependent manner. Sodium selenite significantly reduced the level of apoptosis over the concentration range of 10 nM-1 μM. The concentrations giving best protection were 100-200 nM sodium selenite. Selenomethionine decreased the levels of apoptosis with concentrations between 50 nM-1 μM (Fig 4.4). The optimal concentrations of selenomethionine for decreasing the level of UVB-induced apoptosis were between 50-200 nM.

4.2.2.2 Confirmation of apoptosis using gel electrophoresis.

Primary keratinocytes grown in 10 cm petri dishes, were incubated with either sodium selenite or selenomethionine for 24 hours prior to the media being replaced with PBS and the cells exposed to 600 J/m^2 UVB. The original Se-containing media was then replaced and the cells were incubated for a further 24 hours. The cells were left for 8 hours longer than when using the acridine orange staining method, as DNA cleavage is a late event in the process of apoptosis. The supernatants and the monolayers of cells were then harvested separately, processed and the DNA run on an agarose gel (Chapter 2, section 2.4.3). The gel was then visualised on a UV transilluminator.

As can been seen in Fig 4.5 the supernatants of the irradiated cells contained apoptotic cells, as DNA ladders were present. This method is not a quantitative method, so the relative amounts of ladder can not be quantitatively compared. However it can be seen that the lane with the supernatant from the unirradiated cells does not contain a DNA ladder.*

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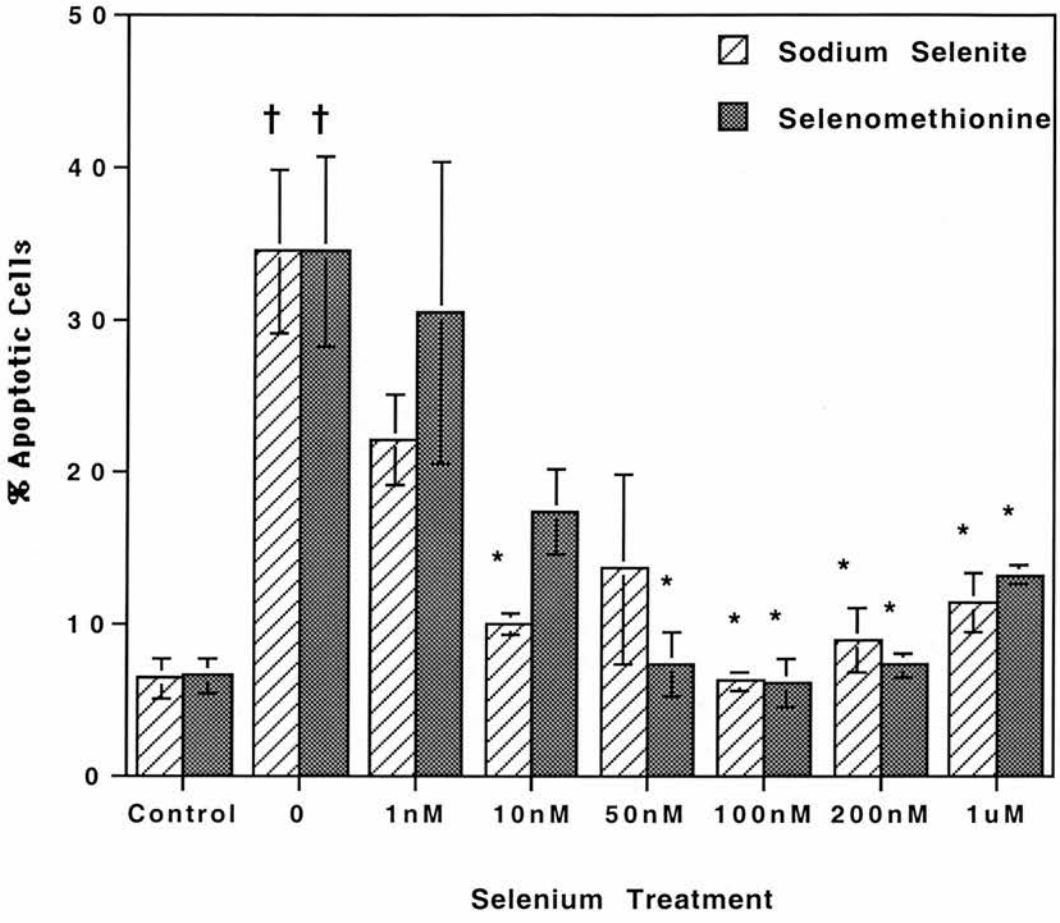
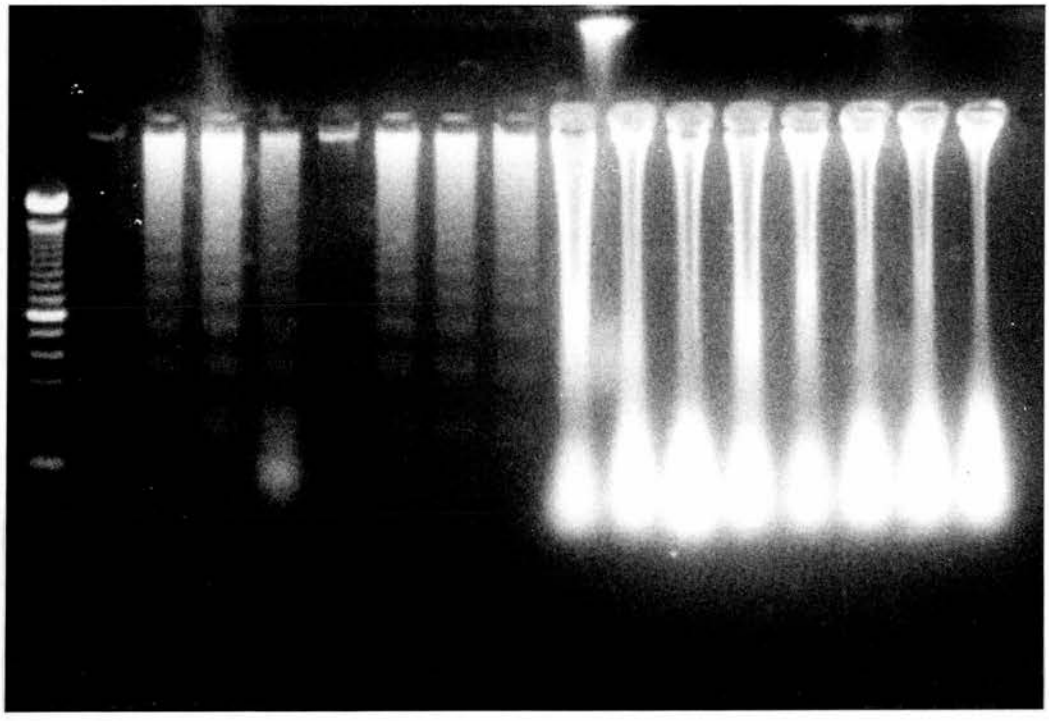
Figure 4.4: Effect of Se on the UVB-induction of apoptosis in primary human keratinocytes.



Cells were supplemented with either sodium selenite or selenomethionine for 24 hours prior to the media being replaced with PBS and the cells exposed to UVB (600 J/m^2). The original Se-containing media was replaced and the cells were incubated for 16 hours. The cells from the monolayers and supernatants were harvested separately, resuspended in PBS, stained with acridine orange and counted using fluorescent microscopy. Control cells were not treated with Se or irradiated. Results are the means of the % apoptosis ± S.E.M, n=6. Significant difference between control cells and irradiated cells with no Se treatment, † = $P<0.05$. Significant difference from irradiated cells with no Se treatment, * = $P<0.05$.

Figure 4.5: Effect of Se and exposure to UVB on the formation of DNA ladders in primary human keratinocytes.



La A B C D E F G H I J K L M N O P

Cells were supplemented with either sodium selenite or selenomethionine for 24 hours prior to the media being replaced with PBS and the cells exposed to UVB (600 J/m^2). The original Se-containing media was replaced and the cells were incubated for 24 hours. The cells from the monolayers and supernatants were harvested separately, lysed, proteinase K and RNase A treated. The DNA was then run on an agarose gel and viewed on a UV transilluminator. 100 base pair ladder is shown (La). Lanes A-H are supernatant cells and Lanes I-P are monolayer cells-: A&I- control unirradiated cells, B&J- irradiated cells with no Se added, C&K- 1 nM sodium selenite, D&L- 10 nM sodium selenite, E&M- 50 nM sodium selenite, F&N- 50nM selenomethionine, G&O- 100 nM selenomethionine and H&P- 200 nM selenomethionine.

The supernatants from cells treated with 10 and 50 nM sodium selenite show less ladder formation than the irradiated control cells. The cells treated with selenomethionine all show DNA ladders.

The monolayers of all of the cells do not show any DNA ladders and only show smears of DNA. The presence of DNA ladders are very difficult to demonstrate, they are only present for a brief period of time during the latter stages of apoptosis. From seven attempts to demonstrate their presence, only two were successful.

4.2.3 Effect of exposure to UVB on the levels of p53 protein found in human primary keratinocytes.

4.2.3.1 Level of protein for p53 measured by western blotting.

Cells were grown to 70% confluence, in six well dishes, the media was replaced with PBS and the cells exposed to doses of UVB between 200-1000 J/m^2. The original media was replaced and the cells were left for 24 hours, harvested into triple lysis buffer and western blotting performed (Chapter 2 section 2.4.4). The blots were then visualised on XOMAT-XAR film using ECL and quantitated on a densitometer.

The first two lanes on the western blot are A431's, a human keratinocyte cell line which express a high level of mutant p53 protein, so were used as a positive control for the p53 protein (Fig 4.6a). The other lanes in Fig 4.6a show the effect of exposure to UVB on the levels of p53 protein in primary keratinocytes, the graph in Fig 4.6b is derived from the western blot in Fig 4.6a.

The protein for p53 increased in a dose dependent manner up to 400 J/m^2 of UVB. However, at doses above 400 J/m^2 the protein level remained high but did not increase further (Fig 4.6 a&b). In further experiments 600 J/m^2 UVB has been used again at 24 hours following exposure to UVB.

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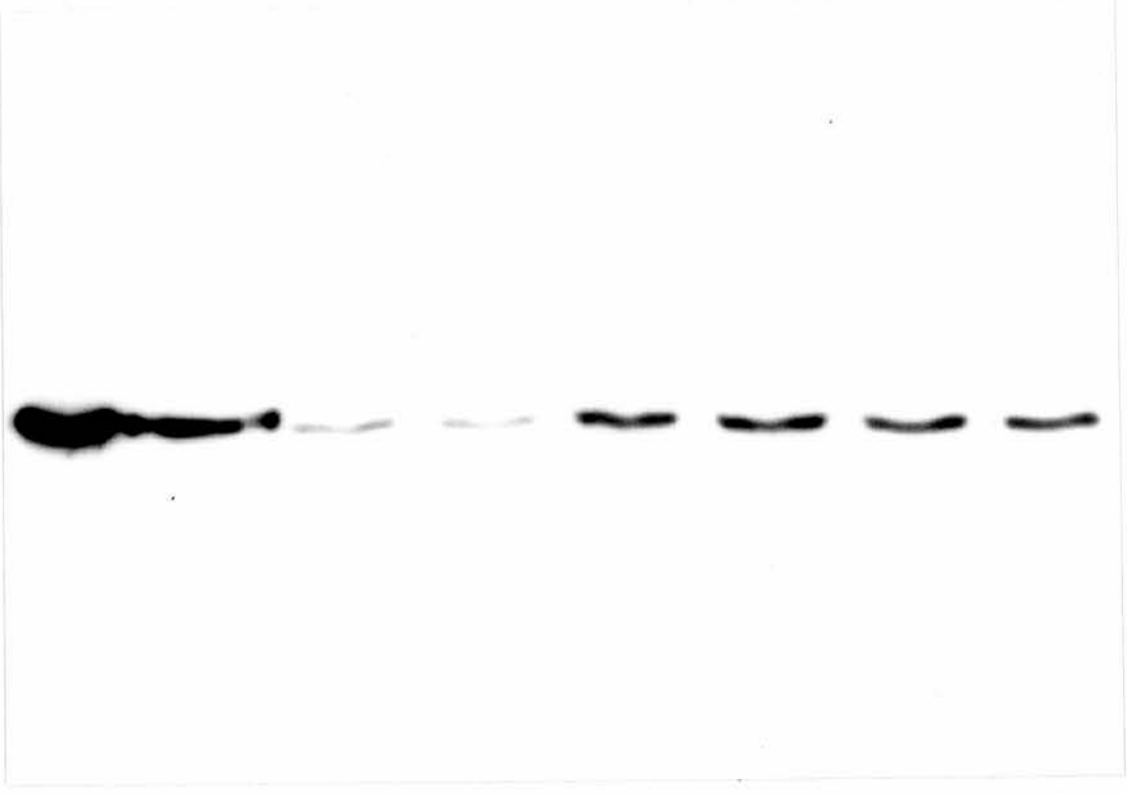
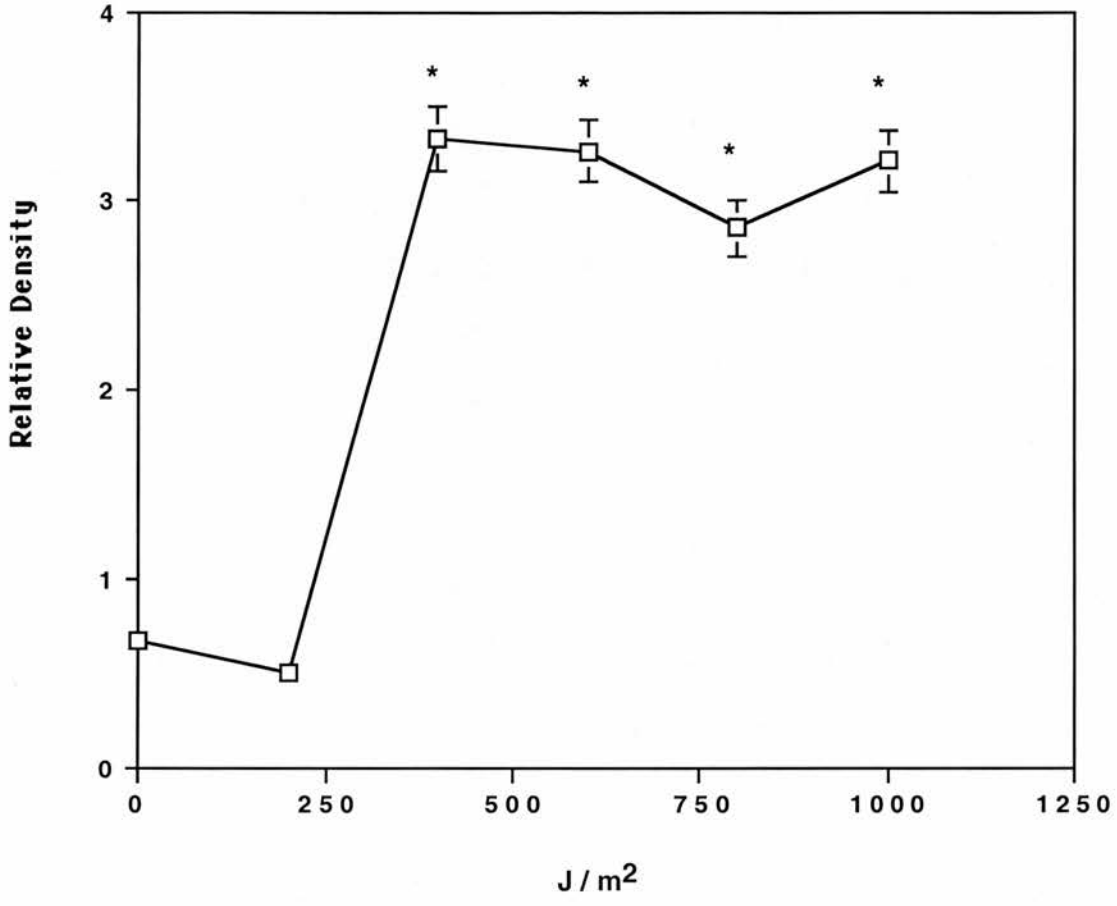
Figure 4.6a: Induction of p53 protein by exposure to UVB, in primary human keratinocytes, measured by western blotting.



A B C D E F G H

Cells were grown to 70% confluence the media was replaced with PBS and the cells exposed to doses of UVB between 200-1000 J/m^2. The original media was replaced and the cells were left for 24 hours, harvested into triple lysis buffer and western blotting performed for p53. The blots were then visualised on to XOMAT-XAR film using ECL. Lane A&B- A431 cells, Lane C- Primary keratinocytes, not exposed to UVB, Lane D-H- Primary keratinocytes exposed to 200, 400, 600, 800 and 1000 J/m^2 UVB.

Figure 4.6b: Induction of p53 protein by exposure to UVB, in primary human keratinocytes, measured by western blotting.



Cells were grown to 70% confluence the media was replaced with PBS and the cells exposed to doses of UVB between 200-1000 J/m^2. The original media was replaced and the cells were left for 24 hours, harvested into triple lysis buffer and western blotting performed for p53. The blots were then visualised onto XOMAT-XAR film using ECL and quantitated on a densitometer. Results are the mean relative densities ± S.E.M, n=2. Significant differences from the 0 hour, unirradiated cells, * = 0.05%.

4.2.3.2 Level of protein for p53 demonstrated by immunostaining.

Primary keratinocytes were grown on coverslips and treated as in section 4.2.3.1. After the 24 hour incubation time following exposure to UVB, the cells were fixed in 1% paraformaldehyde and immunostained for p53 (Chapter 2, Section 2.4.5). They were then studied under the microscope. A scoring system was devised to grade the intensity of staining.

Normal unirradiated keratinocytes showed a faint nuclear staining for p53 (Fig 4.7a). It was found that 600 J/m^2, 24 hours following exposure to UVB demonstrated again the best induction of p53 (Figure 4.7b). The staining for p53 was darker in the irradiated cells and remained nuclear. In the control cells all of the cells show faint staining for p53, then after irradiation this staining intensifies, this made it difficult to quantitate the staining.

Using the scoring system, exposure to UVB induced an increase, in the abundance of p53 protein at low levels in cells exposed to 100-300 J/m^2 (Table 4.1). This increased in strength in cells exposed to 100-400 J/m^2, however the intensity of staining did not increase further at doses of UVB above 400 J/m^2.

4.2.4 Effect of Se on p53 induction by exposure to UVB in primary keratinocytes.

4.2.4.1. Measured by western blotting.

The cells were treated with sodium selenite (1-50 nM) or selenomethionine (50-200 nM) for 24 hours before the media was replaced with PBS and the cells exposed to 600 J/m^2 UVB. The original Se-containing media was returned and the cells incubated for a further 24 hours. The cells were then harvested as before and western blotting carried out for p53 (Chapter 2, section 2.4.4). The experiment was repeated twice and each point was done in duplicate.

The unirradiated control cells express low background levels of the protein for p53. The cells exposed to UVB, but not treated with Se, show a four-fold increase in the abundance of p53 protein. Sodium selenite, did not affect the

UVB-induced increase in the abundance of the protein for p53 at any concentration tested (Fig 4.8).

In the second experiment, using selenomethionine, the irradiated cells show a three fold increase in p53 protein, however due to large variations in the control unirradiated cells, this did not achieve statistical significance (Fig 4.9). Unfortunately, this occurred in both of the duplicate experiments with selenomethionine. However, it can be seen that the selenomethionine had no affect on p53 in irradiated cells at any concentration tested (Fig 4.9).

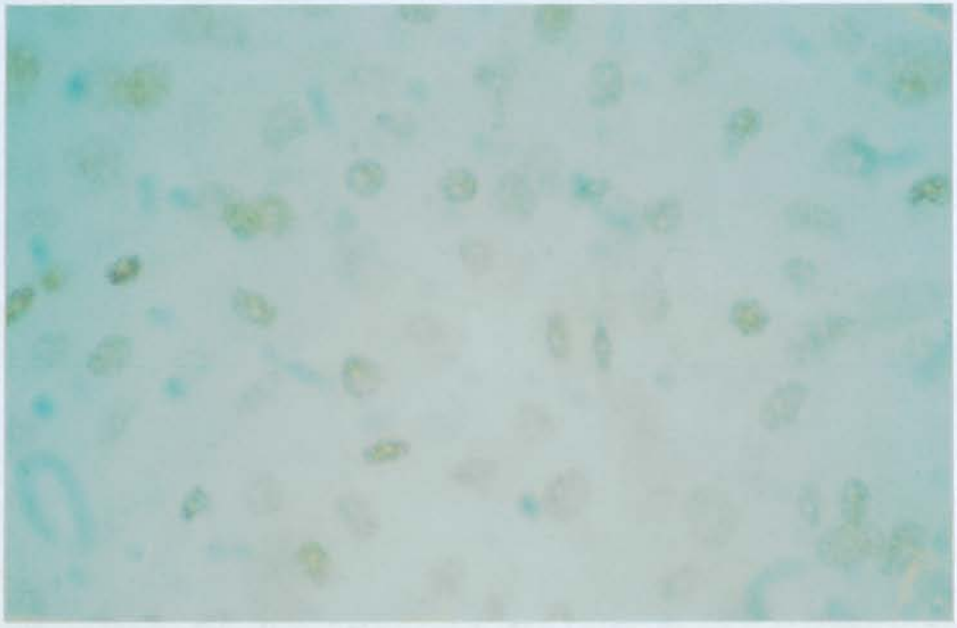
4.2.4.2 Demonstrated using immunostaining.

Primary keratinocytes were grown on coverslips and treated with either sodium selenite or selenomethionine for 24 hours prior to the media being replaced with PBS. The cells were then exposed to 600 J/m^2 UVB, the original Se-containing media was replaced and the cells incubated for a further 24 hours. After the 24 hour incubation, the cells were fixed in 1% paraformaldehyde and immunostained for p53 (Chapter 2, section 2.4.5). They were then studied under the microscope .

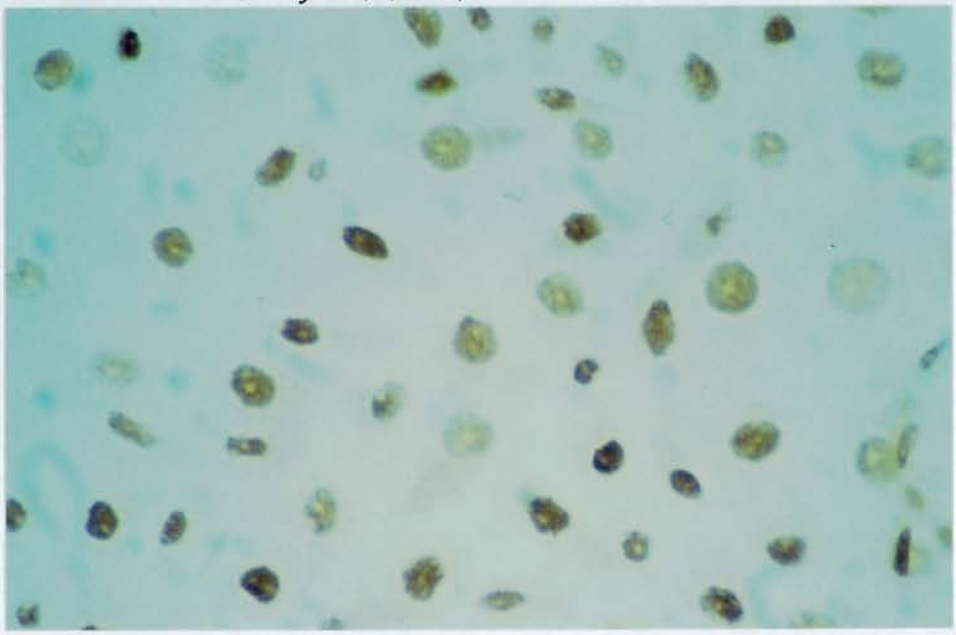
As can be seen from Table 4.2, Se did not greatly alter the intensity of staining for p53. There were indications of slight decreases in staining at 10 nM sodium selenite and 100 and 200 nM selenomethionine. However these were not consistent in all of the experiments, nor were they large decreases. Treatment with Se did not alter the location of the p53 within the cell, it remained nuclear.

Figure 4.7: Induction of p53 protein by exposure to UVB in primary human keratinocytes, demonstrated by immunostaining.

(a) Unirradiated cells (x160).



(b) Irradiated cells (600 J/m^2) (x160).



Primary keratinocytes were grown on coverslips and treated as in section 4.2.4.1. After the 24 hour incubation time following exposure to UVB the cells were fixed in 1% paraformaldehyde and immunostained for p53. The staining intensity was then graded under the microscope.

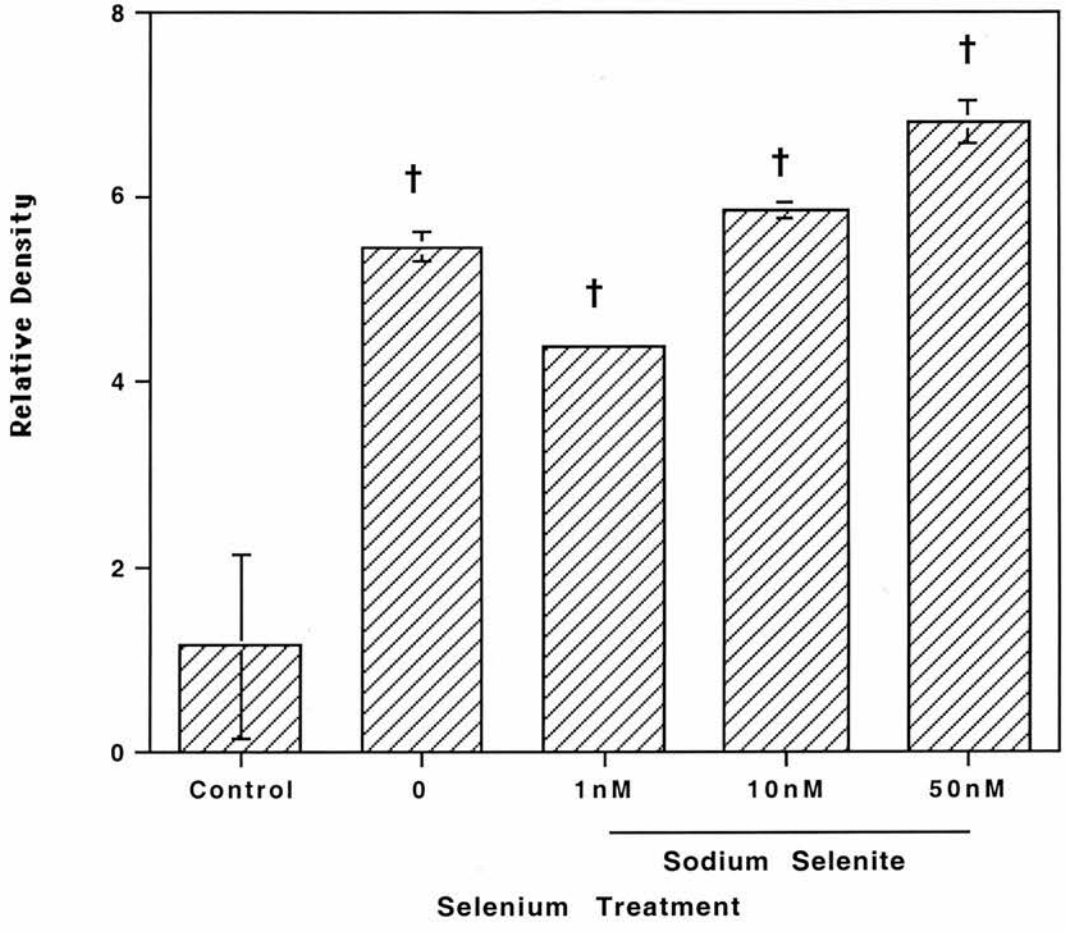
Table 4.1: Induction of p53 protein by exposure to UVB in primary human keratinocytes, measured by immunostaining.

J/m^2 UVB	Intensity of staining
0	+/-
100	+
200	++
300	++
400	+++
500	+++
600	+++

Primary keratinocytes were grown on coverslips and treated as in section 4.2.4.1. After the 24 hour incubation time following exposure to UVB the cells were fixed in 1% paraformaldehyde and immunostained for p53. They were then studied under the microscope, and graded for the intensity of staining.

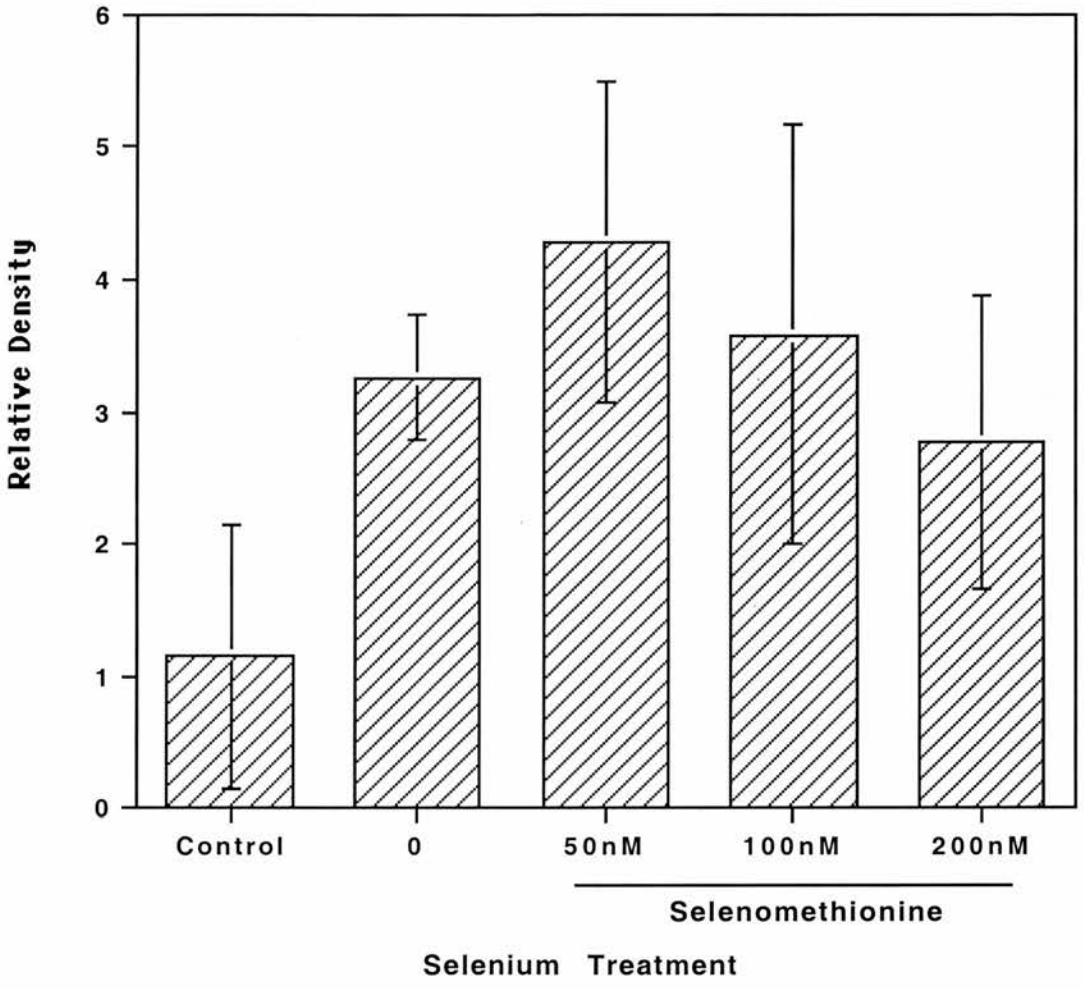
- = no staining
+ = faint staining
++ = stronger staining
+++ = very strong dark brown staining

Figure 4.8: Effect of Se on the UVB-induced increase in p53 protein in primary keratinocytes, measured by western blotting.



The cells were treated with sodium selenite for 24 hours before the media was replaced with PBS and the cells exposed to 600 J/m^2 UVB. The original Se-containing media was returned and the cells incubated for 24 hours. The cells were then harvested into triple lysis buffer and western blotting carried out for p53. The blots were then visualised using ECL and scanned using a densitometer. Control cells were not treated with Se or exposed to UVB. Results are the mean densitometer readings ± S.E.M, n=2. Significant induction compared to 0, unirradiated cells, † = P<0.05%.

Figure 4.9: Effect of Se on the UVB-induced increase in p53 protein in primary keratinocytes, measured by western blotting.



The cells were treated with selenomethionine for 24 hours before the media was replaced with PBS and the cells exposed to 600 J/m^2 UVB. The original Se-containing media was returned and the cells incubated for 24 hours. The cells were then harvested into triple lysis buffer and western blotting carried out for p53. The blots were then visualised using ECL and scanned using a densitometer. Control cells were not treated with Se or exposed to UVB. Results are the mean densitometer readings ± S.E.M, n=2.

Table 4.2: Effect of Se on the UVB-induced increase in the level of p53 protein in primary keratinocytes, measured by immunostaining.

Treatment	Relative intensity of staining (individual experiments)		
Control	+/-	+/-	+
0	+++	++/+++	+++
1nM sodium selenite	+++	+++/++	+++
10nM sodium selenite	+++/++	++	+++
50nM sodium selenite	+++	++/+++	+++
50nM selenomethionine	++	++/+++	+++/++
100nM selenomethionine	++	++	++
200nM selenomethionine	+	++/+	+++

The cells were treated with sodium selenite or selenomethionine for 24 hours before the media was replaced with PBS and the cells exposed to 600 J/m^2 UVB. The original Se-containing media was returned and the cells incubated for 24 hours. The cells were then immunostained for p53. Control cells had not been treated with Se or exposed to UVB. Results are the average intensity of staining, following the key below, n=3.

- = no staining
+ = faint staining
++ = Stronger staining
+++ = Very strong dark brown staining

4.3 Discussion.

Following exposure to UVB, skin cells can undergo cell death. Cell death induced by exposure to UVB was originally considered to be by the process of necrosis. Necrosis is described as a "passive" form of cell death, during which the cell swells and lyses, spilling it's cellular contents into the surrounding tissue and thus induces an inflammatory reaction. However, it is now accepted that at lower doses of UVB a large proportion of the cell death is mediated by apoptosis. Apoptosis in comparison to necrosis is an "active" form of cell death, which is characterised by specific cellular changes. Cells are observed to shrink and fragment into membrane enclosed apoptotic bodies, which are then phagocytosed by other cells. The associated phagocytosis ensures that the cellular contents do not leak, therefore an inflammatory response does not occur (Kerr *et al*, 1972).

At the peak induction of apoptosis by UVB, as much as 30% of the keratinocyte population dies by apoptosis (Baba *et al*, 1996, Cotton and Spandau, 1997). Furthermore the optimum time point for the induction of apoptosis in the skin, has been demonstrated previously to be around 15 hours post exposure to 600 J/m^2 UVB. An optimum time of 15 hours compares well with the results of the present study, were 30% of cells were induced to undergo apoptosis 16 hours after exposure to 600 J/m^2 UVB (Fig 4.1). The level of apoptosis appears to decrease at later time points, this is because *in vivo* the apoptotic bodies would have been phagocytosed by other cells. However, *in vitro* there are no cells to phagocytose the apoptotic cells so over time they eventually lyse and the level of apoptosis appears to decrease. The presence of apoptotic cells was rigorously confirmed by three separate methods. The use of multiple investigative techniques is important when studying cell death, due to the similarities between apoptosis and necrosis. The presence of apoptotic cells was confirmed using acridine orange staining, where cell shrinkage and the subsequent formation of apoptotic bodies could be demonstrated (Fig 4.2). The cellular morphology was then studied by electron microscopy and a series of events leading to apoptosis was followed. It should be noted that in any given cell population cells are found at different stages of apoptosis, due to cell to cell variation in the rate of apoptosis. However cell shrinkage, condensation of the

chromatin, contraction of the nucleus, and formation of apoptotic bodies could all be demonstrated in irradiated keratinocytes.

As shown in Figure 4.1 and 4.4 the unirradiated cell population has a low background level of apoptosis. Therefore apoptosis is one of the mechanisms by which normal, untreated keratinocytes in culture die. There is much evidence to suggest that sunburn cells in the epidermis are apoptotic and that differentiating keratinocytes exhibit many of the features of apoptosis (Maruoka *et al*, 1997). These include the activation of endonucleases and DNA fragmentation (McCall and Cohen, 1991). However there are differences between terminally differentiating and apoptotic cells. Terminally differentiating cells can take days and not hours to die. Also such cells do not fragment and form apoptotic bodies and specific keratin genes are upregulated in differentiating cells (Gandarillas *et al*, 1999). However in culture, normal keratinocytes appear to undergo a low level of apoptosis, whether this is the case *in vivo* is unclear.

There is increasing evidence that ROS are involved in the activation of all the major pathways of apoptosis. Exposure to UV light can induce oxidative stress. Therefore the effect of Se on the UVB-induction of apoptosis was studied. It was found that Se, added as sodium selenite or selenomethionine significantly decreased UVB-induced apoptosis. Both compounds acted in a dose dependent manner (Fig 4.4). Examination of the results showed that sodium selenite was protective at slightly lower concentrations (10 nM) compared to selenomethionine (50 nM). This is in agreement with the overall cell survival experiments reported in Chapter 3, were sodium selenite was again found to be protective at slightly lower concentrations (1 nM) than selenomethionine (10 nM) (Fig 3.3a). The more potent protection offered by sodium selenite is thought to be a consequence of its increased bioactivity, as discussed in Chapter 3. The decrease in apoptosis shown differs from the protection offered against overall cell death. This is because higher concentrations (1 μM) of both compounds were found to decrease the level of apoptosis, than the concentrations which where found to reduce overall cell death (200 nM). It is not known why this should occur, however it maybe due to the fact that lower doses of UVB were used to induce apoptosis. Therefore the cells are incurring less damage and can cope more readily with the extra stress induced by the two seleno-compounds. In

general terms however, the protection from apoptosis for both compounds is very similar to the concentrations which afford overall protection from cell death.

The data in figure 4.5 again demonstrates the protective effects of Se following cell exposure to UVB. As shown, the formation of nucleosomal ladders following UVB-exposure was decreased by pre-treatment of the cells with either 10 or 50 nM sodium selenite. As described in the results section, this feature of apoptosis is considered a late event and is difficult to detect and quantitate. However it does serve as a third method for confirming the presence of apoptosis in keratinocytes following exposure to UVB. Also as presented in figure 4.5, unirradiated cells do not appear to form nucleosomal ladders, as none were detected by this assay. However the sensitivity of the assay may be the limiting factor in detecting any nucleosomal ladder formation in unirradiated cells.

The apoptosis stimulated by the exposure of cells to UVB appears to be induced by the activation of several different pathways. It would appear that the Fas, TNFR and p53 pathways are all involved in UV-induced apoptosis (Gniadecki *et al*, 1997).

One of the mechanisms by which apoptosis is induced following exposure to UVB is via the production of ROS. Apoptosis in keratinocytes following exposure to UVB can be decreased by the addition of the antioxidants such as α-tocopherol (Malorni *et al*, 1996) and glutathione (Godar, 1999). Therefore Se as a component of the antioxidants GPX or TR may have an important role in preventing the induction of apoptosis by ROS. Due to time constraints a full analysis of the three main pathways of apoptosis in keratinocytes following exposure to UVB could not be completed.

Antioxidants (thioredoxin and N-acetylcysteine) can inhibit the TNF-mediated pathway of apoptosis (Matsuda *et al*, 1991; Chang *et al*, 1992). Both TNF-α and TNFR are induced by exposure to UVB and antibodies to TNF-α partially block UVB-induced apoptosis (Schwartz *et al*, 1995). The effect of Se on the UVB-stimulation of TNF-α was studied in detail and is covered in Chapter 6). However it did appear that Se can decrease the UVB-induction of the mRNA and the protein for TNF-α in primary human keratinocytes.

Therefore Se could decrease the induction of apoptosis by TNF-α, in response to UVB by, helping to regulate the cells control of transcription and translation.

Another pathway involved in the control of apoptosis is the Fas/FasL signalling pathway. Exposure to UVB induced Fas and FasL on keratinocytes and it can also induce the Fas molecules to cross link which can induce apoptosis (Aragane *et al*, 1998). Cross- linking of Fas molecules is thought to involve superoxide anions and hydroxyl radicals (Gorman *et al*, 1997). This pathway can be decreased by the addition of antioxidants such as thioredoxin and SOD (Matsuda *et al*, 1991; Hirose *et al*, 1993). The Fas/FasL pathway was not examined in this study, however it does appear to be strongly influenced by ROS and antioxidants do reduce the induction of apoptosis by this mechanism. Therefore it is certainly a candidate mechanism for the reduction of apoptosis by Se treatment following exposure to UVB.

Finally another pathway leading to apoptosis is the p53 pathway. It has been shown that p53 knock out mice have greatly reduced levels of apoptosis in the epidermis following exposure to UVB (Ziegler *et al*, 1994). Moreover reactive oxygen-mediated DNA damage has been shown to lead to the accumulation of p53 (reviewed in Buttke and Sandstrom, 1994). In Chapter 5, Se was found to reduce the formation of oxidative DNA damage. The ability of Se to prevent oxidative DNA damage is of great importance since work done using antioxidants points to a role for oxidative stress in the activation of p53 (Renzing *et al*, 1996). Antioxidants may act by preventing the phosphorylation of p53. The N-terminal domain of p53 requires phosphorylation before the bound mdm-2 is released, the release of mdm-2 stabilises the p53 protein (Midgley and Lane, 1997; Shieh *et al*, 1997). The UV-induced protein kinase JNK-1 has been shown to phosphorylates p53 at the N-terminal (Milne *et al*, 1995). The C-terminal end of p53 also needs to be phosphorylated or acetylated to increase it's ability to bind DNA. Indeed DNA damage induced by exposure to UVB has been shown elicit the phosphorylation of p53 at the C-terminal (Lu *et al*, 1998). It has been demonstrated that the REDOX environment is important for the conformation of the p53 DNA binding site (Sun and Oberley, 1996).

Therefore oxidative stress is important in the activation and conformation of the p53 protein.

The levels of p53 protein were shown to increase 24 hours following exposure to 600 J/m^2 UVB (Fig 4.6a & b). The rise in p53 levels shown here are in agreement with other reported findings in the literature (Hall *et al*, 1993). Selenium did not affect the induction of the p53 protein by UVB. The results suggest that Se does not act by blocking the phosphorylation of the p53 protein at the N-terminus of the protein, where mdm-2 binds. For there to be an increase in the abundance of p53, phosphorylation of the N-terminus must occur allowing the mdm-2 to be released and Se did not block the increase in the protein. The upregulation of the p53 protein is reported to be the result of post translational modification and not to an increase in mRNA levels. However from this study it cannot be distinguished whether the p53 protein displays an increase in DNA binding ability or stimulates increased transcription of the genes for effector proteins in the p53 pathway. It may be that Se alters the transcriptional activation of the p53 target proteins. Also Se may exert its effects by increasing p53's transcription of DNA repair pathway proteins or cell cycle arrest proteins such as $p21^{WAF/CIP1}$ and GADD45. This could be augmented by decreasing the activation of the apoptotic genes which p53 regulates such as Bax and Bcl-2. Bcl-2 has antioxidant properties (reviewed in Kroemer, 1997). It is known to inhibit apoptosis by preventing the release of cytochrome c from mitochondria. Cytochrome c can act as a co-factor to activate Apaf-1 which activates caspase 9, an important pro-apoptotic effector.

It has recently been shown that caspase 3, in particular is a key player in the DNA fragmentation process and other morphological changes associated with apoptosis. Selenite (100 nM) pre-treatment has been shown to decrease the level of UVB-induced apoptosis, DNA fragmentation and caspase 3 activation (Park *et al*, 2000). The inhibition of caspase activity may be a major mechanism by which primary keratinocytes are protected from UVB-induced apoptosis.

An interesting recent discovery is that p53 can activate the antioxidant selenoprotein GPX (Tan *et al*, 1999). The biological importance of this is not known, however it may be that p53 activates this gene in order to decrease

the oxidative stress within the cell. Any activation of GPX by p53 would increase the possibility of the cell recovering from exposure to UVB. If p53 activated the GPX gene in conditions of low Se then the protein would not be produced optimally. However, supplementation of the cells with extra Se would allow the cells to produce more functional GPX, thus increasing the protection of the cell. The effect of Se supplementation in primary keratinocytes, on the levels of cGPX and phGPX are covered in Chapter 8.

4.4 Further Work.

There is a great deal of further work which could be carried out to discover how exactly Se decreases the level of apoptosis induced by exposure to UVB. It would be of interest to study the Fas-mediated pathway of apoptosis and determine if Se decreases Fas clustering on the surface of cells. Also of interest is the role of the down stream effector proteins in this pathway. An investigation into the role Se has on the recruitment of FADD, MORT and other effectors to the Fas pathway would be very informative.

It could be determined if Se alters the protein levels of any members of the Bcl-2 family thereby rescuing cells from apoptosis. The levels of activation of the effector caspases is also a candidate for further examination. Similarly work could be carried out on the TNF-α pathway to dissect out the mode of Se's protective effect.

A great deal of work needs to be carried out on the p53 pathway, to determine which downstream genes are activated by UVB and if Se alters the transcriptional state of these genes i.e. Bcl-2, Bax, $p21^{WAF/CIP1}$ and GADD45. An examination of the phosphorylation pattern of p53 in the presence or absence of Se would allow greater understanding of the mechanism of p53 induction. Finally electrophoresis mobility shift assays could be utilised to determine the DNA binding capability of the p53 protein, in nuclear extracts with and without Se supplementation.

Chapter 5

The Effect of Se on the Formation and Repair of UVB-Induced DNA Damage in the Skin.

5.1 Introduction.

5.1.1 Mechanism of Photocarcinogenesis.

As discussed in detail in Chapter 1 it has been established that natural sunlight is a complete human skin carcinogen, affecting all three stages of photocarcinogenesis: initiation, promotion and progression (Brash *et al*, 1996).

At the level of initiation, exposure to UV radiation induces DNA damage, which may cause mutations within cellular DNA. Exposure to UV radiation causes DNA mutations and failure to repair these genetic alterations may lead to unrestrained growth and tumour formation. The importance of UVB as an initiator of tumourigenesis is thought to result from it's ability to cause mutations in genes which control the cell cycle. Examples of these genes are proto-oncogenes such as ras or tumour suppressor genes such as p53 (van der Schroeff *et al*, 1990: Brash *et al*, 1991). There is increasing evidence to link DNA damage to skin carcinogenesis, the most compelling evidence is that patients with the hereditary condition *xeroderma pigmentosum* are 2000 times more likely than unaffected individuals to develop skin carcinomas (Lambert *et al*, 1995). Patients with *xeroderma pigmentosum* are characteristically defective in nucleotide excision repair pathways (Cleaver, 1969).

5.1.2 Ultraviolet Radiation-induced DNA damage.

The effects of UV radiation are due to the absorption of radiant energy by an appropriate molecule (chromophore). The wavelengths of UV that are carcinogenic for human cells correspond to the absorption spectrum of DNA (Sutherland *et al*, 1980). DNA absorbs UV radiation in the range 230-300 nm.

UV radiation may damage DNA directly, as observed with shorter wavelengths such as UVC or UVB. Alternatively, damage may be caused indirectly by longer wavelength UVA, via a photosensitiser and the formation of reactive oxygen species (Friedberg *et al*, 1995). The different lesions are discussed in detail in Chapter 1, however a short summary is included below:

Direct DNA damage includes the formation of cyclopyrimidine dimers (CPDs) which are the predominant solar UV photoproduct. The 6-4 pyrimidine-pyrimidone photoproduct is the second most common form of direct DNA damage caused by solar UV radiation.

There is substantial evidence linking direct types of DNA damage to skin photocarcinogenesis. Analysis of DNA from human basal and squamous carcinomas, has revealed that the majority of mutations occur at dipyrimidine sites.

Indirect forms of DNA damage induced by exposure to UV radiation include oxidised or hydrated pyrimidines (cytosine photohydrates and thymine glycols), oxidised purines (8-hydroxyguanosine) (8-OHdg), single-strand breaks and DNA protein cross-links (Jen *et al*, 1997). There is some evidence that indirect DNA damage may be involved in carcinogenesis (van der Scroeff *et al*, 1990; Nishigori *et al*, 1994).

The hydroxyl radical appears to be important in the formation of oxidative DNA damage (Peak and Peak, 1990). Singlet oxygen has also been shown to be involved in the formation of 8-OHdg. Oxidised DNA damage has been reported to occur after exposure of keratinocytes to H_2O_2, although the damage is thought to be mediated by the formation of hydroxyl radical (Beehler *et al*, 1992; reviewed in Thomas *et al*, 1998). The damage caused by exposure of cells to H_2O_2 includes the formation of oxidised pyrimidines and single strand breaks (Collins *et al*, 1995; Duthie and Collins, 1997). Lipid peroxidation has also been reported to mediate the formation of 8-OHdg damage (Park and Floyd, 1992). The mutated base 8-OHdg is formed in a dose-dependent manner following exposure of the skin to UVB (Hattori-Nakakuki *et al*, 1994; Hattori *et al*, 1996).

5.1.3 Repair of UV-induced DNA damage.

Efficient repair of UV photoproducts is a critical early step in the prevention of skin cancer. DNA photoproducts will lead to skin cancer only if they persist long enough in basal cell keratinocytes, to become fixed as permanent mutations in subsequent rounds of cell division.

Base excision repair removes areas of damaged DNA either as single bases, or as short oligonucleotides. Nucleotide excision repair (NER), removes both indirect and direct types of DNA damage. The NER is a general repair mechanism that removes bulky DNA lesions causing major helical distortions. These repair mechanisms and other more novel pathways of DNA repair are covered in more detail in Chapter 1.

5.1.4 Protection from DNA damage by antioxidants.

There is an increasing amount of evidence which suggests that treatment with antioxidants decreases the levels of oxidative damage to DNA. Supplementation of human skin fibroblasts with N-acetylcysteine for 4 hours or zinc for 1 week decreases the DNA damage induced by exposure to UVA. The type of damage which was decreased was immediate damage such as single strand breaks and alkali-labile sites (Emonet-Piccardi *et al*, 1998). Glutathione has been reported to decrease the formation of 8-OHdg, in a cell free system following exposure to ionising radiation (Fischer-Nielsen *et al*, 1994).

5.1.5 Protection from DNA damage by supplementation with Se.

Sodium selenite (600 nM) was found to decrease the formation of single strand breaks and alkali-labile sites in skin fibroblasts, after exposure to UVA (Emonet-Piccardi *et al*, 1998). Pre-treatment with sodium selenite (5 μM) for 48 hours prior to exposure to UVB (500 J/m^2) also decreases the formation of 8-OHdg sites in mouse keratinocytes (Stewart *et al*, 1996). In the same report, ascorbic acid and vitamin E also prevented the formation of 8-OHdg lesions following exposure to UVB. Single strand breaks induced by exposure of cells to H_2O_2 can be decreased by supplementation of cells with

50 nM sodium selenite (Leist *et al*, 1996). The data presented here suggests that Se can reduce the formation of oxidative DNA damage.

5.1.6 The Comet assay.

The comet assay (also known as single-cell gel electrophoresis) is a rapid and sensitive method of measuring DNA strand breaks in mammalian cells. This assay was first described by Ostling and Johanson (Ostling and Johanson, 1984) and has been developed by Singh and Olive (Singh *et al*, 1988; Olive *et al*, 1990). Methods and applications of this assay have been extensively reviewed (McKelvey-Martin *et al*, 1993; Fairbairn *et al*, 1995; Collins *et al*, 1997). During the assay cells are embedded in agarose, lysed, electrophoresed, and stained before DNA damage is visualised using fluorescent microscopy (Fig 5.1). Breaks in the duplex DNA molecule release its complex super-coiling and the free DNA migrates toward the anode, so that the cell resembles a comet with a brightly fluorescent head and a tail streaming away from it.

The comet assay can distinguish between breakage of the sugar-phosphate backbone and/or damage to DNA bases. Using time course experiments the assay can distinguish between direct strand breakage and breakage occurring as a result of the repair process. Breakage of the DNA due to the repair process can be measured by the addition of DNA synthesis inhibitors, which allow the recognition and incision stages of excision repair to proceed, but which inhibit DNA resynthesis, causing an accumulation of single strand breaks. Some commonly used inhibitors include; Arabinosyl cytosine (ARA C) and aphidicolin which act by becoming incorporated into the DNA strand thereby distorting its shape so that the DNA polymerase detaches. Another commonly used DNA synthesis inhibitor is hydroxyurea, which inhibits ribonucleotide reductase activity, therefore blocking the production of all deoxyribonucleotides and hence DNA synthesis (Fig 5.2). The comet assay can be easily modified to detect several types of strand breaks and discriminate between different types of DNA damage by using purified DNA repair enzymes (Gedik *et al*, 1992; Collins *et al*, 1993; Collins *et al*, 1996). The Comet assay has been used *in vivo* and *in vitro* to assess DNA damage and repair induced by a variety of physical factors such as: UV radiation (Arlett *et al*, 1993; Wollons *et al*, 1997), chemical agents, cytokines (Delaney *et*

al, 1993), exercise (Hartmann *et al*, 1994), smoking and ageing (Betti *et al*, 1994; Piperakis *et al*, 1998).

Other applications of the comet assay include: determination of genotoxicity, determination of an individual's DNA repair capacity, DNA damage and repair studies, detection of apoptosis and biomonitoring.

5.1.7 DNA repair enzymes.

In this Chapter DNA repair enzymes have been used in conjunction with the comet assay to investigate the different types of DNA damage that can be induced by UVB. These are base excision repair enzymes that incise and cleave sites containing specific types of DNA damage. These enzymes incise the DNA at their specific recognition sites to produce single strand breaks in DNA.

5.1.8 Foramidopyrimidine-DNA Glycosylase (FaPy-glycosylase).

FaPy-glycosylase is an enzyme which catalyses the excision of open-imidazole ring-structures from DNA and has been isolated from extracts of E.Coli or mammalian cells. FaPy-glycosylase is a small zinc-containing protein (31 kDa), which recognises 8-OHdg lesions in DNA (Fig 5.3). The 8-OHdg lesion is ubiquitous and can result from both endogenous and exogenous damage to DNA by ROS.

5.1.9 T4 Endonuclease V (pyrimidine dimer DNA glycosylase).

Bacteriophage T4 is the only bacterial virus known to encode an enzyme specifically for the excision of pyrimidine dimers in DNA (Fig 5.3). The T4 Endonuclease V is a small monomeric protein of 18 kDa with no requirement for cofactors. The enzyme catalyses the selective incision of DNA at the site of pyrimidine dimer formation, and is absolutely specific for CPDs in DNA. The gene product, T4 endonuclease V has been partially purified from T4 infected E.Coli cells (Friedberg, 1971). When applied to the skin in liposomes, it increases the rate of dimer removal from irradiated mouse skin (Yarosh *et al*, 1992).

The aims of this study were to:

- Study the level of excision repair sites formed in primary keratinocyte DNA by exposure to UVB.

- Investigate the effect of Se on the formation of excision repair sites in keratinocyte DNA.

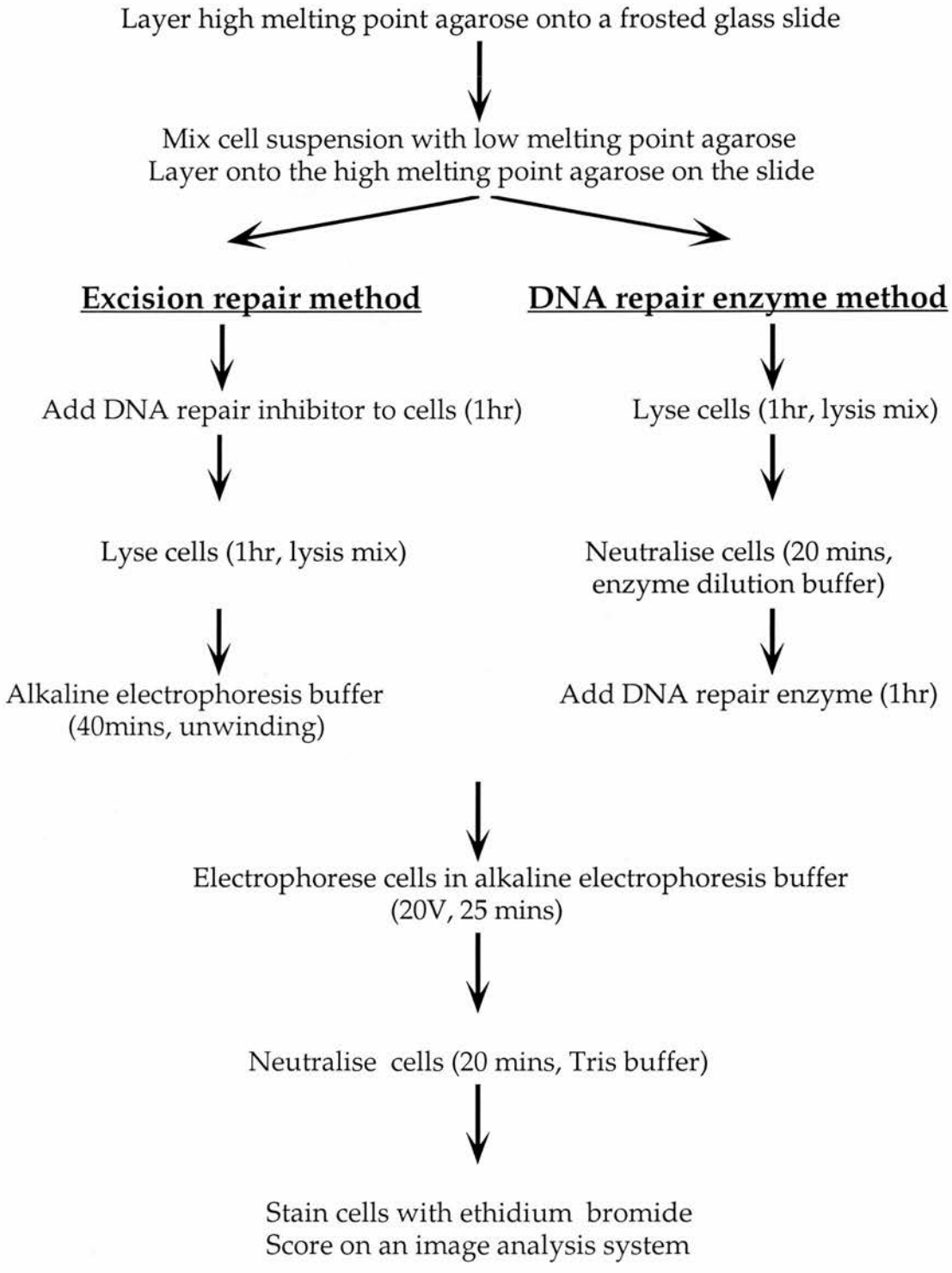
- Monitor the UVB-induction of CPDs and their rate of repair in primary keratinocytes and primary skin fibroblasts.

- Investigate the effect that Se supplementation has on the formation and rate of repair of CPDs in primary keratinocytes.

- Monitor the UVB-induction of 8-OHdg lesions and their rate of repair in primary keratinocytes.

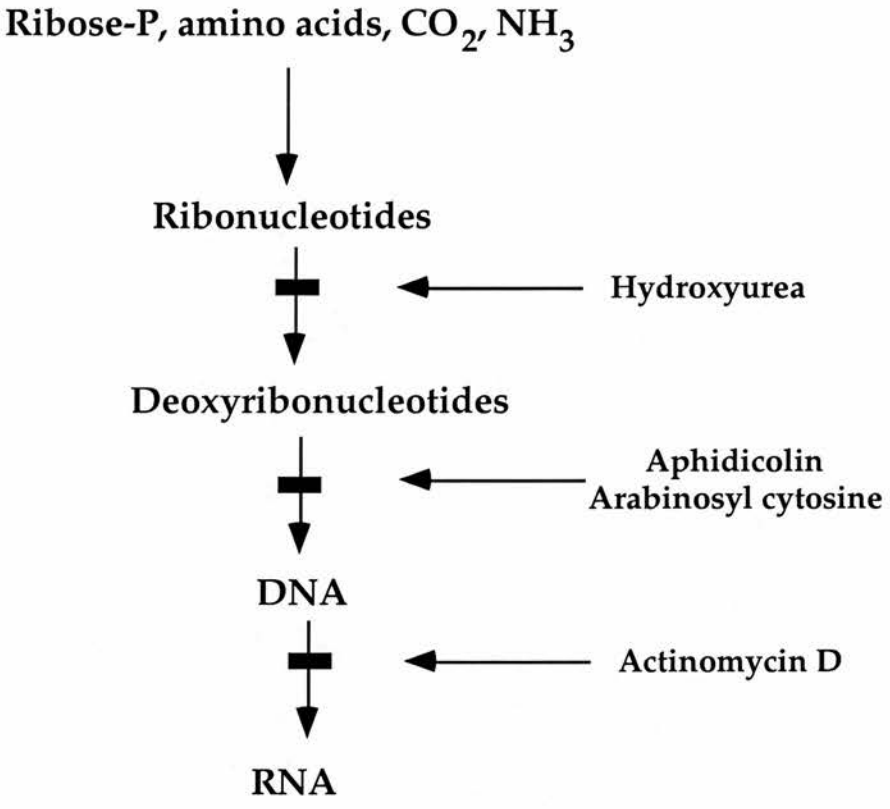
- Study the effect that Se supplementation has on the formation of 8-OHdg lesions in primary keratinocytes.

Figure 5.1: Schematic of the comet assay method.



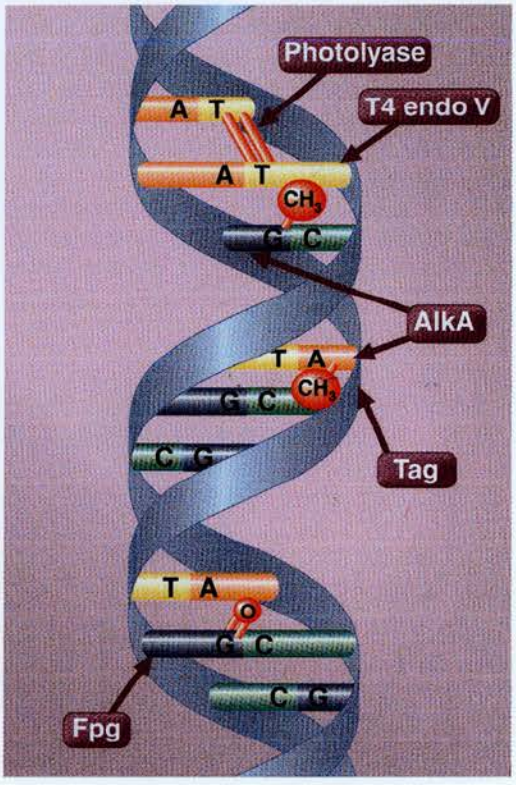
N.B.- Entire method is carried out in the dark.

Figure 5.2: Effect of different DNA synthesis inhibitors.



Sites of action of a selection of DNA synthesis inhibitors

Figure 5.3 Sites of action of the repair enzymes used during the comet assay



The main repair enzymes of interest are the T4 endonuclease V (T4 endo V) enzyme which recognises cyclobutane dimers and FaPy-glycolase (Fpg) which recognises 8-hydroxyguanine

5.2 Methods and Results.

The experiments in this study were carried out by T. Rafferty at the MRC Cell Mutation Unit, University of Sussex, Brighton. The UVB source used was Westinghouse FS-20 broad spectrum UVB lamps. The emission spectra is similar to the Philips TL-20W/12 lamps which were employed in the majority of the work described in this thesis. The FS-20 lamps emit slightly less UVC than the TL-12 lamps. Emission spectra for both lamps are shown in Chapter 2. Very low levels of UVB were required to induce DNA damage, therefore Nunclon tissue culture dishes were used as filters to reduce the outputs of the lamps. The emission spectra for the lamps with and without filters are shown in Chapter 2.

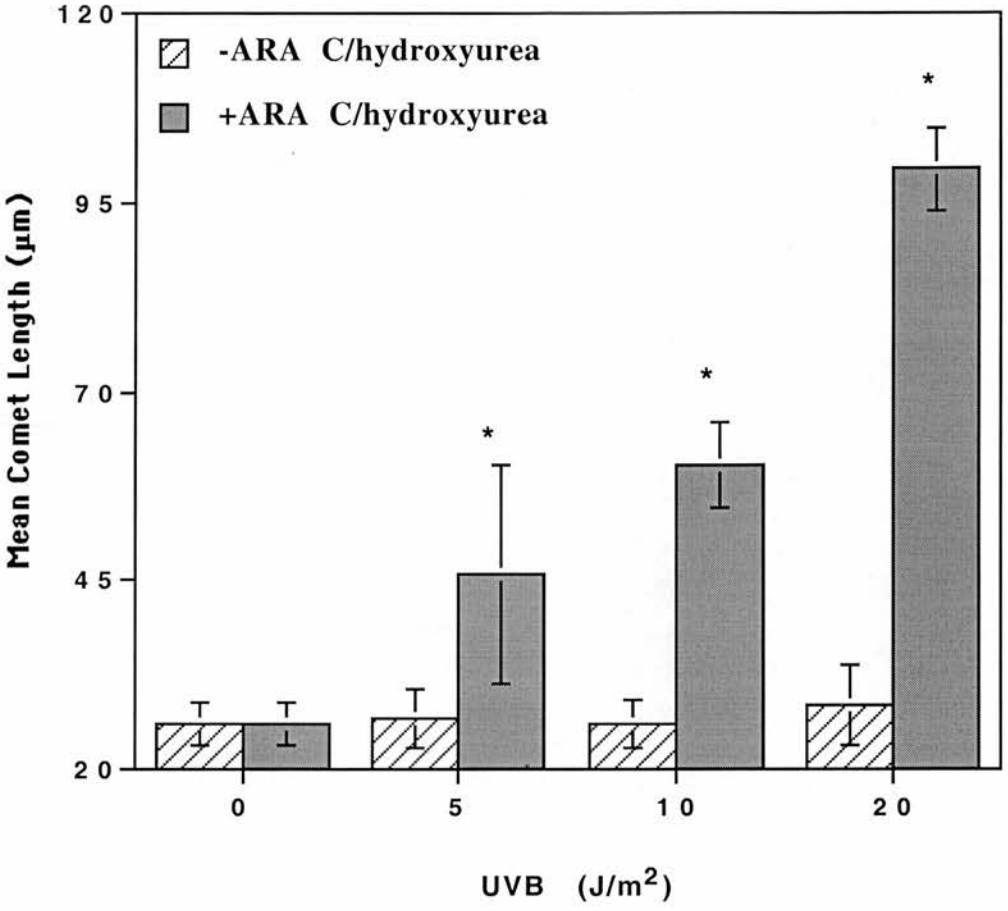
5.2.1 Induction of excision repair sites in primary keratinocytes.

Primary keratinocytes were harvested and $2x10^4$ cells were mixed with low melting point agarose and layered onto slides. Once the agarose had cooled and solidified the cells were exposed to increasing doses of filtered UVB (5-20 J/m^2). DNA synthesis inhibitors were then added to the cells; ARA C (100 μM) and hydroxyurea (10 mM) and the cells were incubated for 1 hour. The slides were then processed using the excision repair comet assay method, which is shown in Fig 5.1 and described in full in Chapter 2, section 2.5.1 and 2.5.2. The comets were then scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment. The experiment was carried out in full twice. A full set of control slides were also counted, which had no ARA C and hydroxyurea treatment, confirming that the DNA strand breaks were excision repair breaks and not single strand breaks.

Damage which required excision repair was formed when primary keratinocytes were exposed to UVB (Fig 5.4). The damage was formed in a dose-dependent manner. There was substantial DNA damage at 5-10 J/m^2 which increased greatly at 20 J/m^2. In subsequent experiments 5 J/m^2 was used to induce damage. The control cells with no ARA C and hydroxyurea treatment, did not show any signs of comet formation, although they had

Continued on page 5-12

Figure 5.4: Formation of excision repair sites in primary keratinocytes exposed to UVB.



Primary keratinocytes were harvested and $2x10^4$ cells, were mixed with low melting point agarose, layered onto slides and cells exposed to filtered UVB (5-20 J/m^2). DNA synthesis inhibitors were then added to the slides, ARA C (100 µM) and hydroxyurea (10 mM) and the cells incubated for 1 hour. The DNA synthesis inhibitors were added together to the slides. The slides were then processed using the excision repair comet assay method and comets scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment. Results are the mean comet lengths ± S.D., n=100. * = significant increase in comet length, $p<0.05$.

been exposed to UVB, thus confirming that no non-specific DNA damage was measured. Furthermore unirradiated cells which were exposed to ARA C/hydroxyurea also had no detectable DNA damage either.

5.2.2 Effect of Se pre-treatment on excision repair sites in primary keratinocytes.

Primary keratinocytes were supplemented with either 50 nM sodium selenite or 200 nM selenomethionine, for 24 hours prior to being harvested and processed as in section 5.2.1, with cells exposed to 5 J/m^2 filtered UVB. The cells were then processed via the excision repair comet assay as described in Chapter 2, section 2.5.2. The experiment was carried out with duplicate samples, five times.

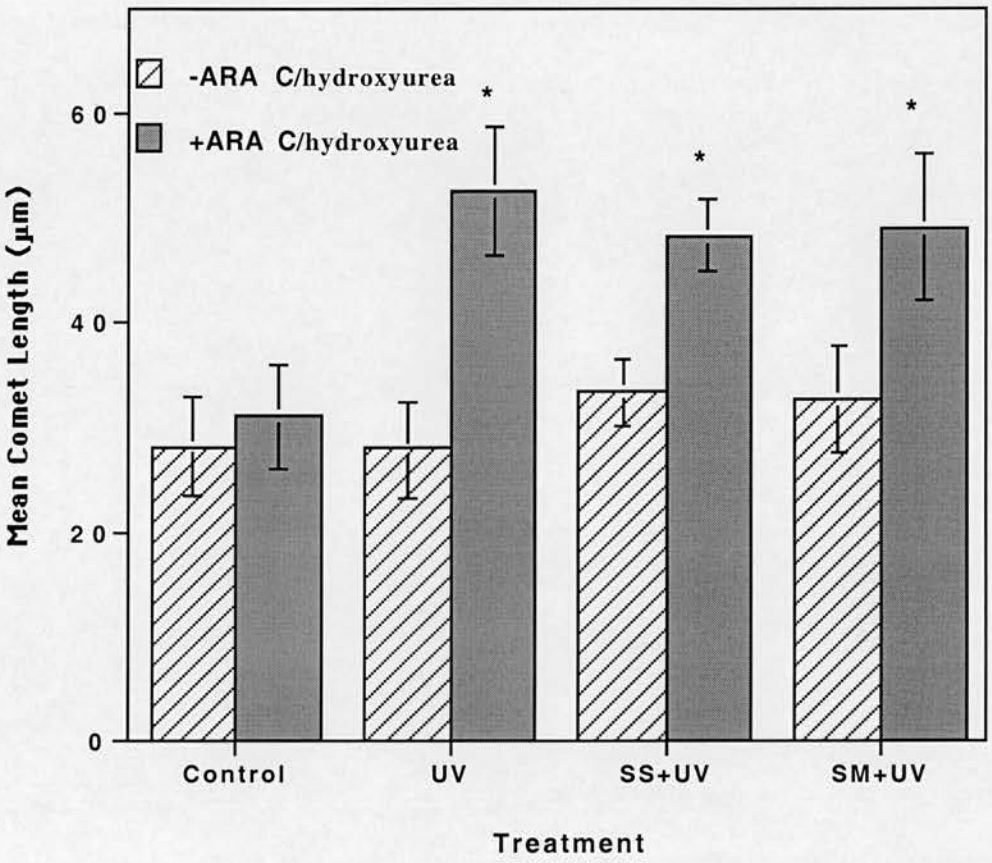
Pre-treatment with Se did not alter the formation of excisable DNA damage (Fig 5.5). The cells untreated with ARA C/hydroxyurea, did not show any signs of DNA damage and neither did the unirradiated ARA C/hydroxyurea treated cells. Primary keratinocytes were also treated with Se only, to ascertain if Se alone induced DNA damage. Neither Se compound (50 nM sodium selenite or 200 nM selenomethionine) induced DNA-damage (results not shown).

5.2.3 Formation of CPDs in primary keratinocytes.

Primary keratinocytes were harvested and $2x10^4$ cells, were mixed with low melting point agarose and layered onto slides. Once the agarose had solidified, the cells were exposed to increasing doses of filtered UVB (1-5 J/m^2). The cells were then processed via the DNA repair enzyme comet method which is described in Chapter 2, section 2.5.3 and is illustrated in Fig 5.1. The enzyme used was 50 μL/slide of T4 endonuclease V (5-20 μg/ml), kindly supplied by L. Mullenders, Leiden, The Netherlands. A full set of slides were processed without the enzyme to confirm that there was no non-specific DNA damage measured by the comet assay. The comets were then scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment and the experiment was repeated twice. *

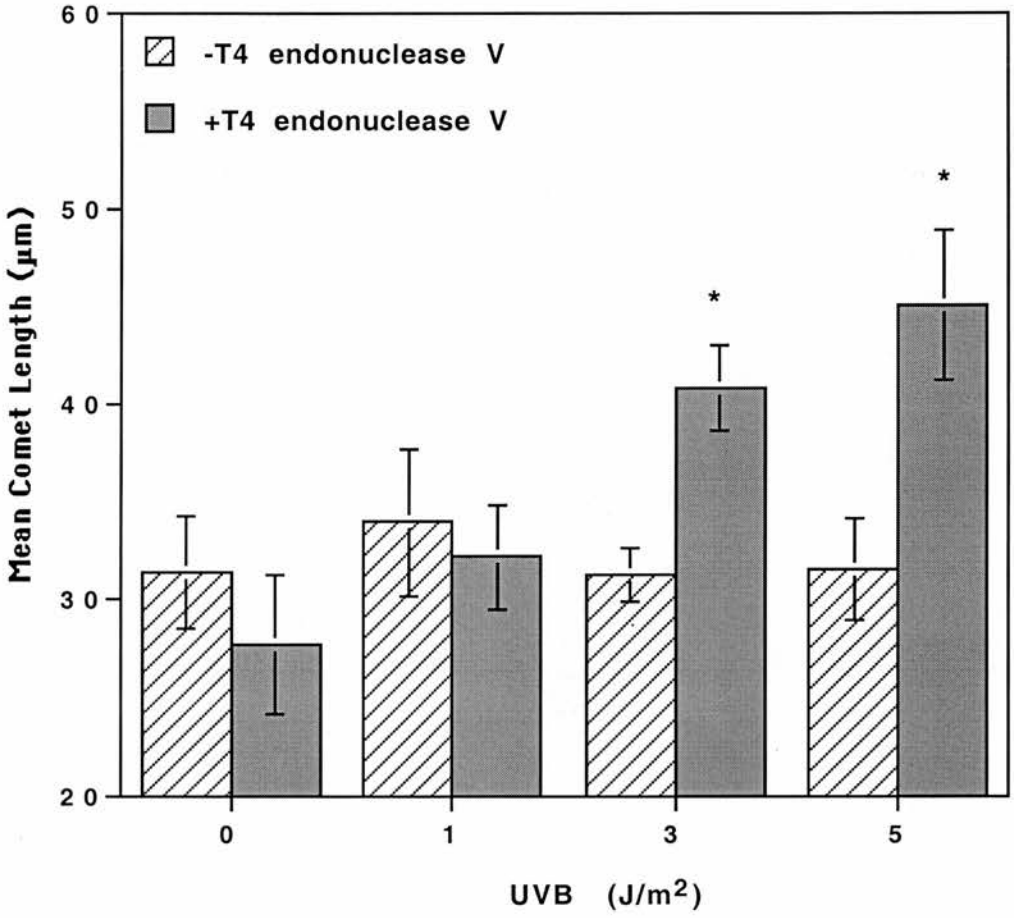
Continued on page 5-15

Figure 5.5: Effect of Se on excision repair in primary keratinocytes exposed to UVB.



Primary keratinocytes were pre-treated with 50 nM sodium selenite (SS) or 200 nM selenomethionine (SM) for 24 hours prior to the cells being harvested, $2x10^4$ cells were mixed with low melting point agarose and layered onto slides. The cells were then exposed to filtered UVB (5 J/m^2). DNA synthesis inhibitors were then added to the slides, ARA C (100 µM)/ hydroxyurea (10 mM) and the cells incubated for 1 hour. The slides were then processed using the excision repair comet assay method and comets scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment. Control cells were not exposed to UVB or Se. Results are the mean comet lengths ± S.D., n=100. * = significant increase in comet length, $p<0.05$.

Figure 5.6: Formation of CPDs in primary keratinocytes exposed to UVB.



Primary keratinocytes were harvested and $2x10^4$ cells were mixed with low melting point agarose, layered onto slides and cells exposed to filtered UVB (1-5 J/m^2). The cells were then processed via the DNA repair enzyme comet method. The enzyme used was 50 µL of T4 endonuclease V (5-20 µg/mL). The comets were scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment. Results are the mean comet lengths ± S.D., n = 100. * = significant increase in comet length, $p<0.05$.

Very low doses of UVB were required to form CPDs in primary keratinocyte DNA (Fig 5.6). Exposure to 3 J/m^2 UVB, induced the formation of CPDs and again the level of DNA damage increased with increasing UV exposure. In subsequent experiments 5 J/m^2 UVB, was chosen as the stimulus. The control cells which were not treated with T4 endonuclease V, did not show any background DNA damage, nor did the irradiated cells which had been enzyme treated.

5.2.4 Effect of Se pre-treatment on the formation of CPDs in primary keratinocytes.

Primary keratinocytes were supplemented with either 50 nM sodium selenite or 200 nM selenomethionine for 24 hours, prior to being harvested and processed as in section 5.2.3. The cells were exposed to 5 J/m^2 filtered UVB. The cells were then processed using the T4 endonuclease comet assay, method from Chapter 2, section 2.5.3. There were duplicate slides per treatment and the experiment was repeated six times.

Se pre-treatment did not affect the initial formation of thymidine dimers in primary keratinocyte DNA (Fig 5.7). The control cells which were not exposed to T4 endonuclease V and the unirradiated enzyme treated cells did not exhibit any signs of DNA damage. Cells which were treated with the seleno-compounds alone and not exposed to UVB irradiation, showed no signs of DNA damage. Therefore neither compound stimulated the formation of CPDs (results not shown).

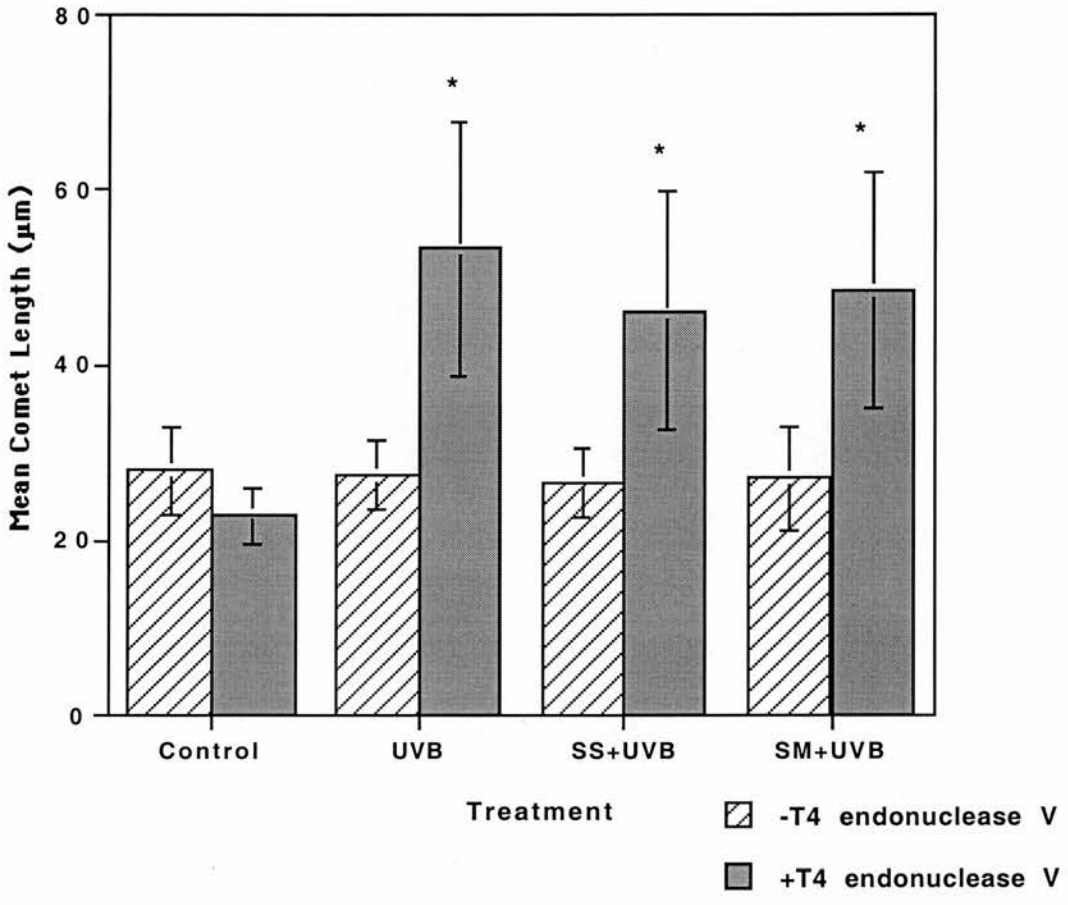
5.2.5 Effect of Se pre-treatment on the repair of CPDs in primary keratinocytes.

Primary keratinocytes were grown in individual 3 cm petri dishes and pre-treated with either 50 nM sodium selenite, or 200 nM selenomethionine for 24 hours. The media was then replaced with 1 ml PBS, and the cells exposed to UVB (5 J/m^2), through the bottoms of the tissue culture dishes (this was to decrease the output of the lamps in order that low levels of UVB were used

*

Continued on page 5-17

Figure 5.7: Effect of Se on the formation of CPDs in primary keratinocytes exposed to UVB.



Primary keratinocytes were pre-treated with sodium selenite (50 nM) or selenomethionine (200 nM) for 24 hours prior to the cells being harvested and $2x10^4$ cells were mixed with low melting point agarose, layered onto slides. The cells were then exposed to filtered UVB (5 J/m^2). The cells were then treated with T4 endonuclease V repair enzyme and processed via the comet method. The comets were scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment. Control cells were not exposed to UVB or Se. Results are the mean comet lengths ± S.D., n = 100. * = significant increase in comet length, $p<0.05$.

to treat the cells). The original Se-containing media was then replaced and the cells were harvested, at various time points following irradiation. Once harvested $2x10^4$ cells were mixed with low melting point agarose and layered onto slides. Once the agarose had solidified, the slides were processed via the T4 endonuclease V repair enzyme comet method, which is described in Chapter 2, section 2.5.3. A full set of slides were again processed without the enzyme to confirm that there was no non-specific DNA damage measured by the comet assay. The comets were then scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment and the experiment was repeated three times.

Selenium did not alter the rate of repair of CPDs in irradiated primary keratinocyte DNA (Fig 5.8). Some examples of fluorescent comets are demonstrated in Figs 5.10a-d. The control cells are compact and there are no comets present (Fig 5.10a). The irradiated cells in comparison have long tails which stream out from the cells (Fig 5.10b). Cells which have been incubated following exposure to UVB to allow time for any DNA repair to occur, display shorter comets. The cells in Fig 5.10c show cells which have been incubated for 2 hours, following exposure to UVB and Fig 5.10d cells which have been incubated for 3 hours. The comets present in Fig 5.10c and d are very short as the majority of the DNA CPDs have been repaired.

Although Se treatment did not alter the rate of repair of CPDs, in primary keratinocytes the experiments did infer that primary keratinocytes repair CPDs rapidly, with most of the damage repaired within 3 hours. The rapid repair of the CPDs can be more clearly seen in Fig 5.9. The results presented in Fig 5.9 come from the same experiment as in Fig 5.8, however the data concerning Se pre-treatment has been removed for clarity. As the primary keratinocytes repaired the CPDs so quickly, it was decided to compare the rate of repair of CPDs in primary keratinocytes and fibroblasts.

5.2.6 Rate of repair of CPDs in primary fibroblasts.

The fibroblasts were grown, irradiated (5 J/m^2) and processed using the T4 endonuclease V comet assay, in the same manner as the keratinocytes in section 5.2.5. Again there were duplicate slides per treatment and the experiment was carried out twice.

It can be observed in Fig 5.11 that fibroblasts are slower at repairing CPDs compared to primary keratinocytes (Fig 5.9). The damage appears to increase slightly at 6 hours and then to decrease slowly over 24 hours until most of the damage is repaired by 48 hours post exposure to UVB.

5.2.7 Formation of 8-OHdg sites in primary keratinocytes exposed to UVB.

Primary keratinocytes were harvested and $2x10^4$ cells were mixed with low melting point agarose and layered onto slides. Once the agarose had solidified the cells were exposed to increasing doses of filtered UVB (250-750 J/m^2). The cells were then incubated with the FaPy-glycosylase enzyme and subjected to the comet assay, which is described in Chapter 2, section 2.5.4. The enzyme used was FaPy-glycosylase, kindly supplied by Dr. A. Collins, Rowett Institute, Aberdeen, UK. The concentration of enzyme used is specific for each batch of enzyme. A full set of slides were again processed without the enzyme, to confirm that there was no non-specific DNA damage measured by the comet assay. The comets were then scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment and the experiment was repeated twice.

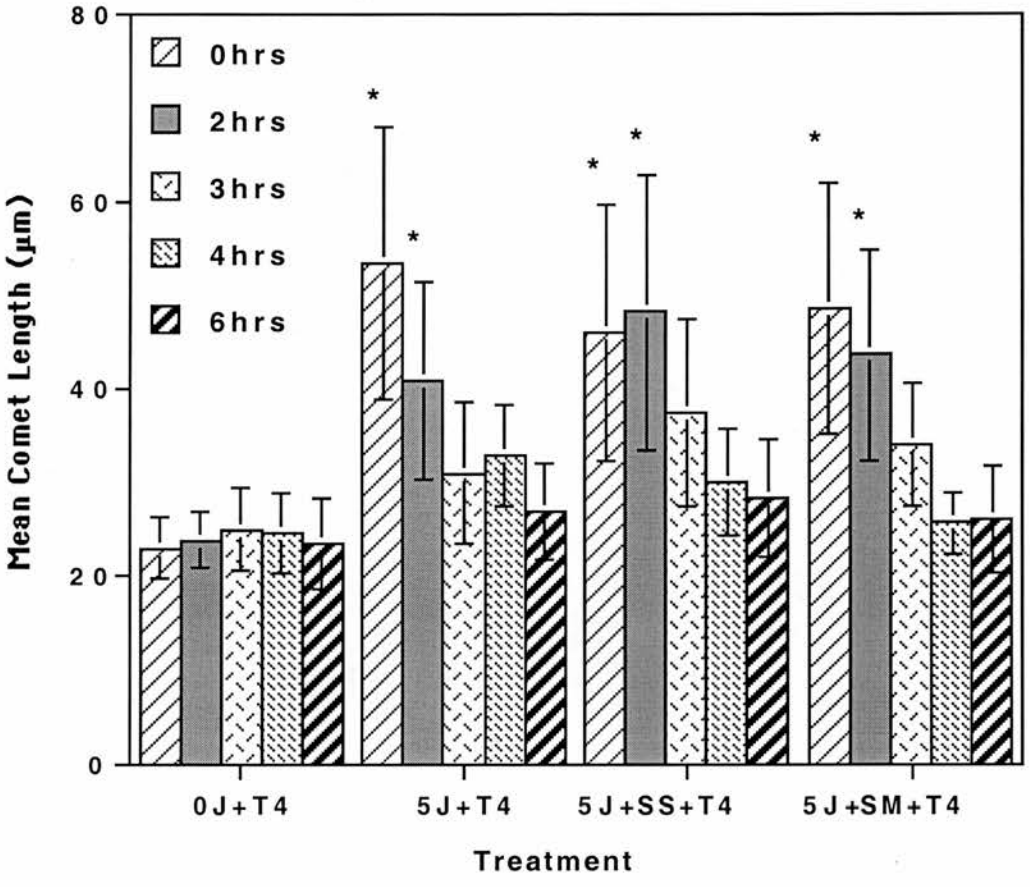
Exposure to UVB induced the formation of 8-OHdg sites in keratinocyte DNA (Fig 5.12). The level of DNA damage induced increased in a dose-dependent manner. The damage present was first significantly induced at 250 J/m^2 and increased at doses up to 750 J/m^2. There was no detectable background DNA damage in the unirradiated cells or in the cells not treated with enzyme. In subsequent experiments a UVB dose of 500 J/m^2 was used.

5.2.8 Effect of Se on the formation of 8-hydroxyguanine sites in primary keratinocytes exposed to UVB.

Primary keratinocytes were pre-treated with either 50 nM sodium selenite or 200 nM selenomethionine for 24 hours prior to being harvested and *

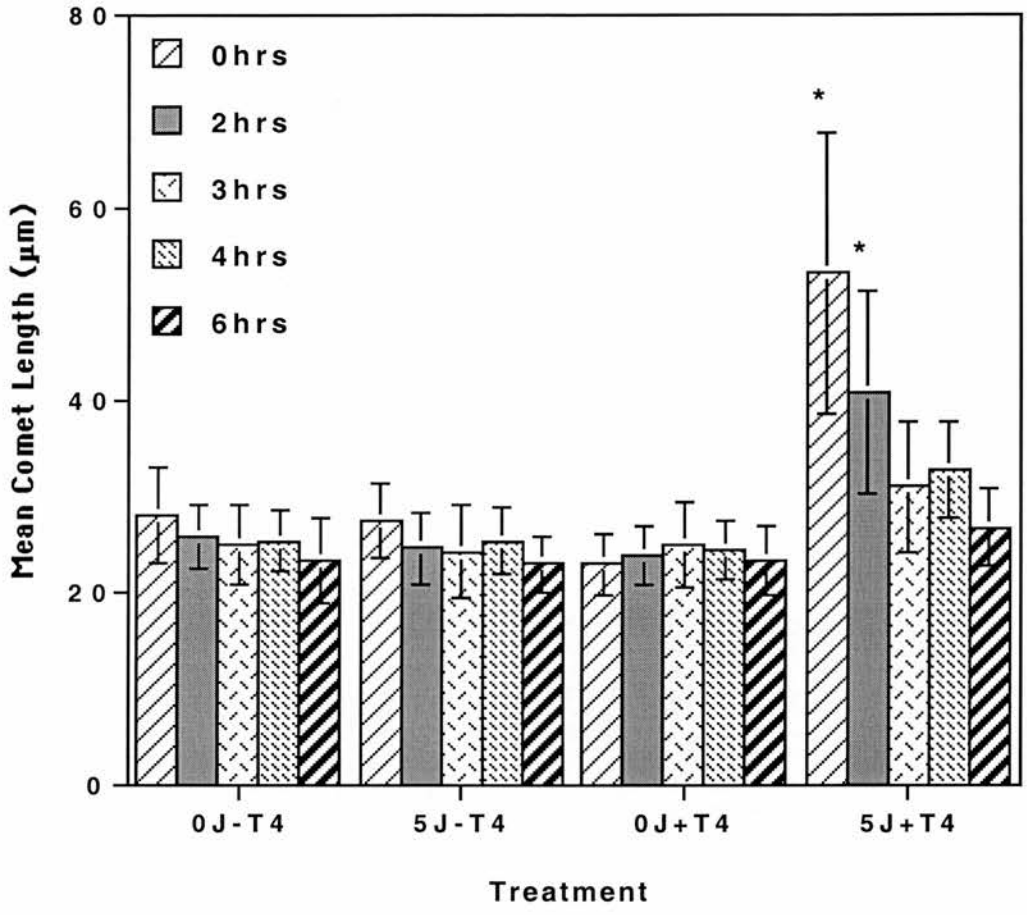
Continued on page 5-24

Figure 5.8: Effect of Se on rate of repair CPDs in primary keratinocytes exposed to UVB.



Primary keratinocytes were grown in 3 cm^2 petri dishes and pre-treated with either 50 nM sodium selenite (SS) or 200 nM selenomethionine (SM) for 24 hours prior to the media being replaced with 1 ml PBS. The cells were then exposed to UVB (5 J/m^2), through the bottoms of the tissue culture dishes. The original Se-containing media was then replaced and the cells harvested at various time points following irradiation. Once harvested $2x10^4$ cells were mixed with low melting point agarose, layered onto slides and incubated with T4 endonuclease V (T4) repair enzyme prior to the comet assay. The comets were then scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment. Results are the mean comet lengths ± S.D, n = 100. * = significant increase in comet length, $p<0.05$.

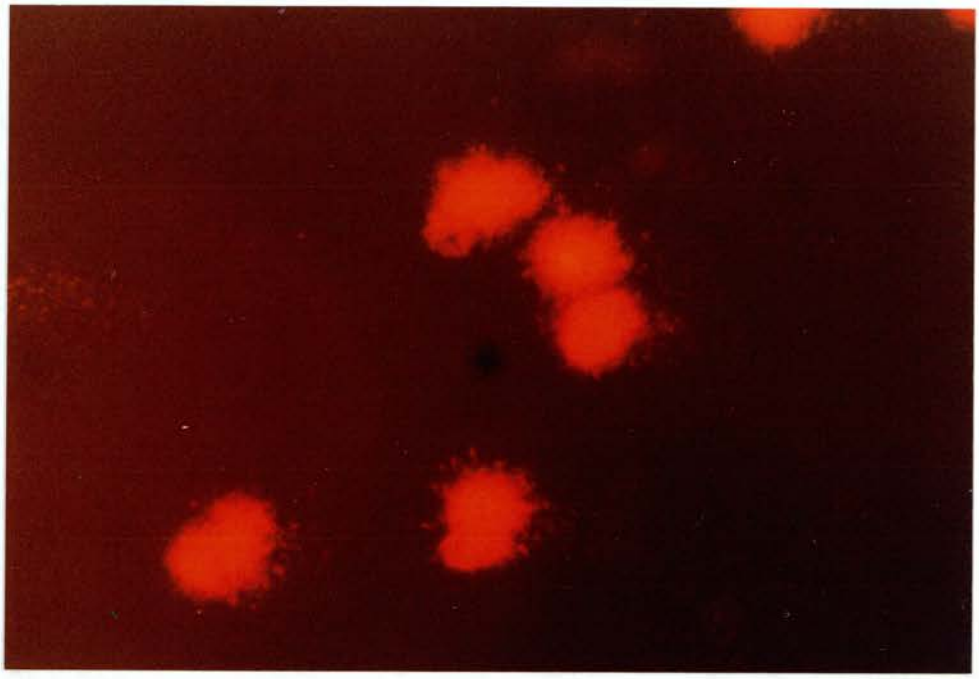
Figure 5.9: Rate of repair of CPDs in primary keratinocytes exposed to UVB.



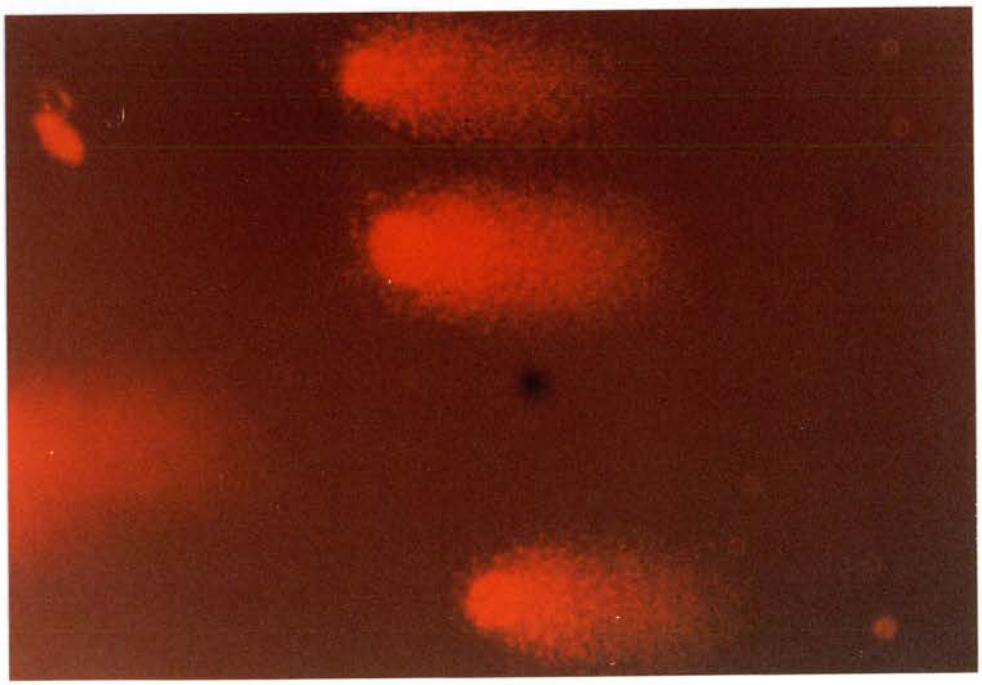
Primary keratinocytes were grown in 3 cm^2 petri dishes and the media was replaced with 1 ml PBS. The cells were then exposed to UVB (5 J/m^2), through the bottoms of the tissue culture dishes. The original media was then replaced and the cells harvested at various time points following irradiation. Once harvested $2x10^4$ cells were mixed with low melting point agarose, layered onto slides and incubated with T4 endonuclease V (T4) repair enzyme prior to the comet assay. The comets were then scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment. Results are the mean comet lengths ± S.D, n = 100. * = significant increase in comet length, $p<0.05$.

Figure 5.10: Visualisation of comet formation during the T4 endonuclease V repair assay using primary keratinocytes exposed to UVB.

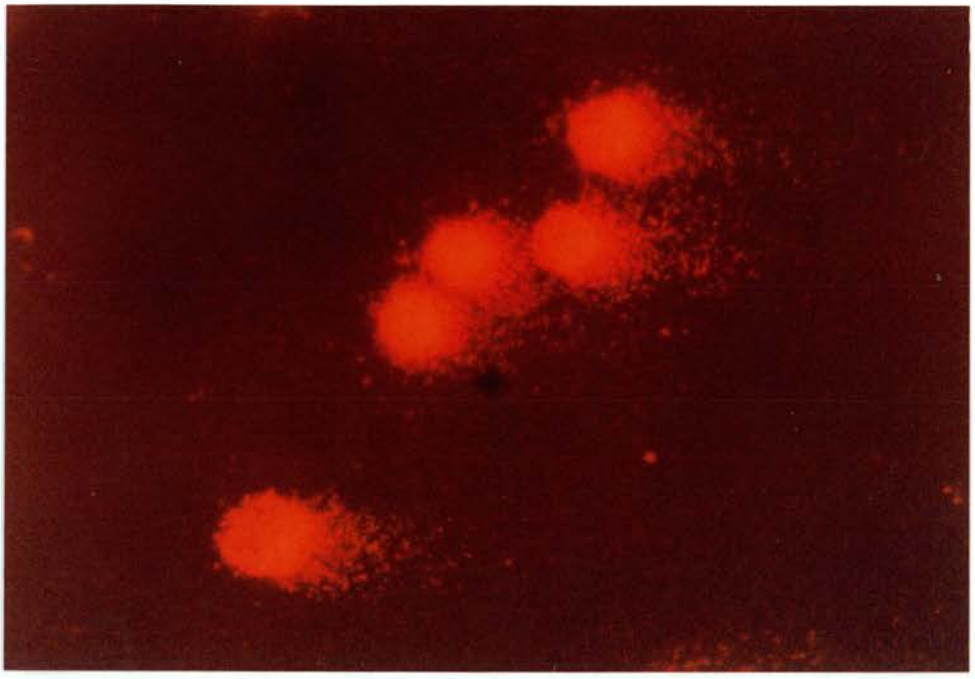
(a) Control cells (x160).



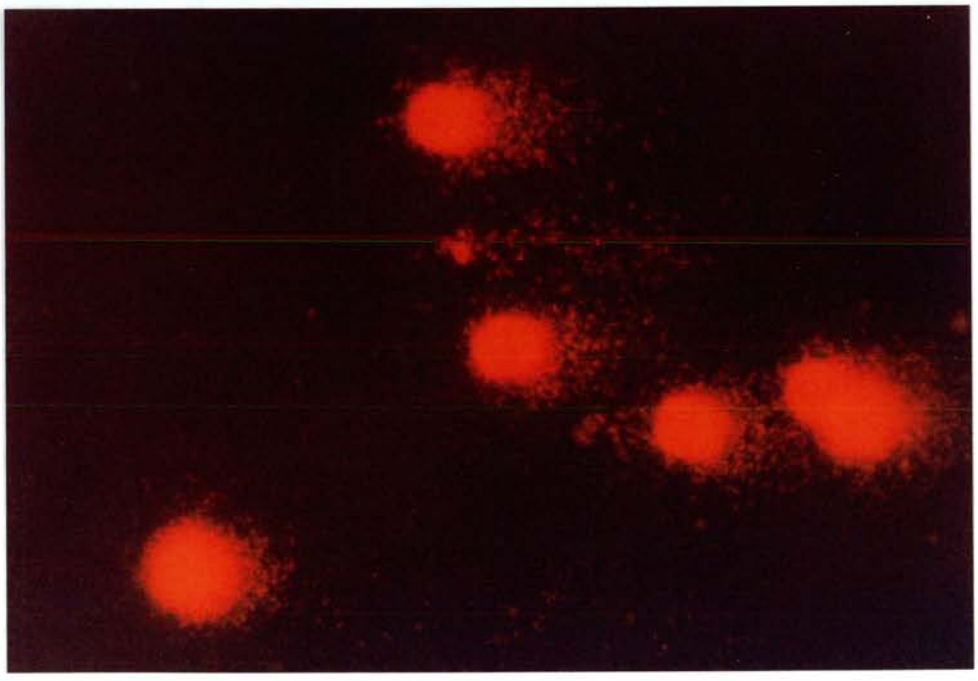
(b) Irradiated cells, 0 hrs post exposure to UVB (5 J/m^2) (x160).



(c) Irradiated cells, 2 hours post exposure to UVB (5 J/m^2) (x160).

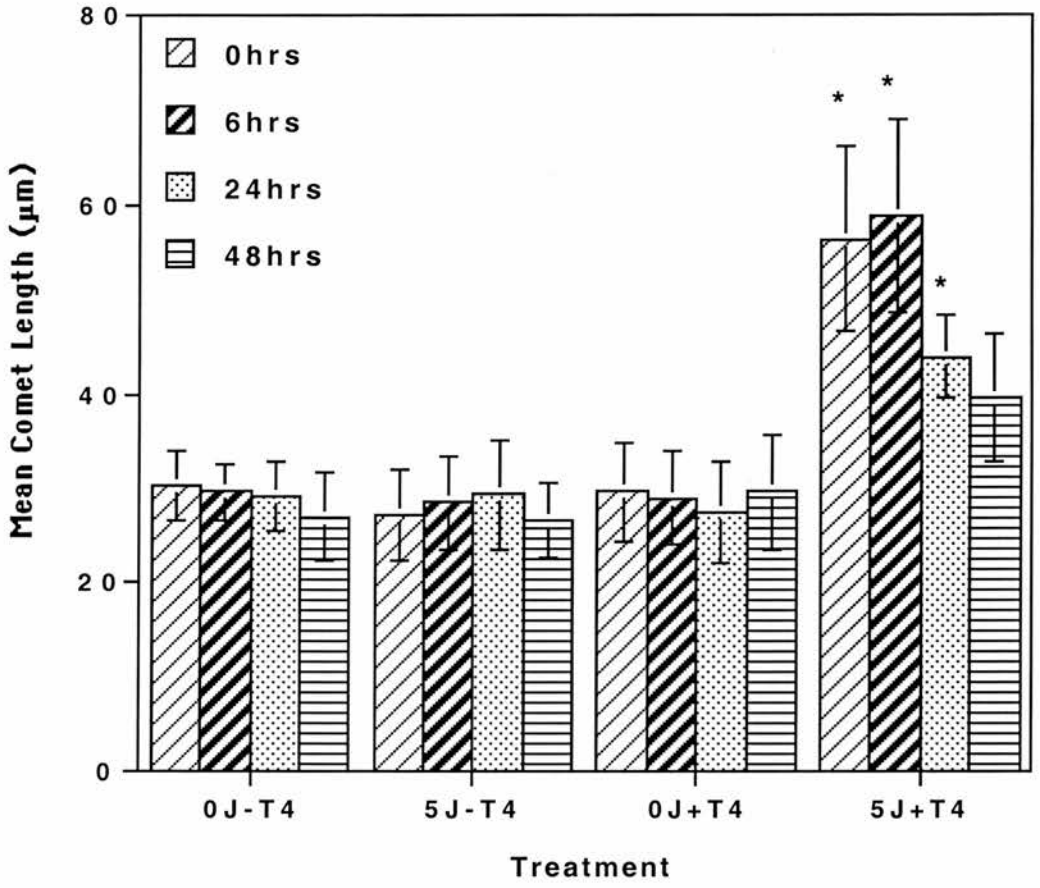


(b) Irradiated cells, 3 hours post exposure to UVB (5 J/m^2) (x160).



Cells were stained with ethidium bromide and visualised using fluorescent microscopy. The typical structure of the head and tail of the comet can be seen. The cell body is the "head" of the comet with the DNA streaming out (to the right) of the cell to form the "tail" of the comet.

Figure 5.11: Rate of repair of CPDs in primary fibroblasts exposed to UVB.



Primary fibroblasts were grown 3 cm^2 petri dishes, then the media was replaced with 1 ml PBS and the cells were exposed to UVB (5 J/m^2), through the bottoms of the tissue culture dishes. The original media was then replaced and the cells were harvested at various time points following irradiation. Once harvested $2x10^4$ cells were mixed with low melting point agarose, layered onto slides and incubated with T4 endonuclease repair enzyme prior to the comet assay. The comets were then scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment. Results are the mean comet lengths ± S.D., n = 100. * = significant increase in comet length, $p<0.05$.

processed as in section 5.2.7, the cells were exposed to 500 J/m^2 filtered UVB.

Pre-treatment with sodium selenite or selenomethionine prevented the formation of 8-OHdg sites in the DNA, following exposure to UVB (Fig 5.13a and b). Both seleno-compounds decreased the formation of 8-OHdg sites, to levels equivalent to those found in the unirradiated control cells (Fig 13 a and b). Again the unirradiated cells and the cells not treated with enzyme showed no background levels of DNA damage.

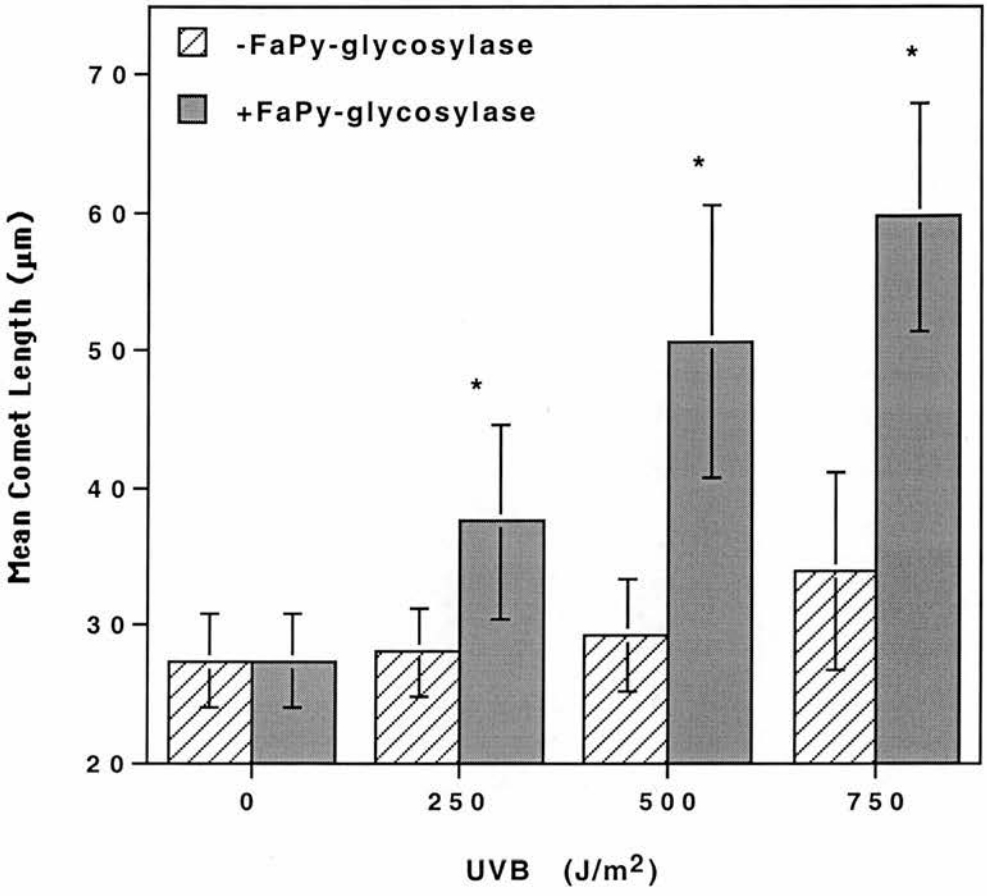
5.2.9 Rate of repair of 8-OHdg sites in primary keratinocytes.

Primary keratinocytes were grown in 3 cm petri dishes, prior to the media being replaced with 1 ml PBS. The cells were then exposed to UVB (150-500 J/m^2), through the bottom of the tissue culture dishes. The original media was then replaced and the cells were harvested at various time points following irradiation. Once harvested, $2x10^4$ cells were mixed with low melting point agarose and layered onto slides. Once the agarose had solidified, the slides were then processed via the FaPy-glycosylase repair comet assay method, which is described in Chapter 2, section 2.5.4. A full set of slides were again processed without enzyme, to confirm that no non-specific DNA damage measured by the comet assay. The comets were then scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment.

It appears from Fig 5.14 that 8-OHdg sites in irradiated DNA are not repaired even 48 hours following exposure to UVB. However, it cannot be ascertained if these are genuine 8-OHdg sites, as the control cells which were not treated with FaPy-glycosylase also showed signs of DNA damage. It appears that following exposure to 350 J/m^2 UVB, or more the cells accumulate secondary DNA damage, which increases with time. Therefore damage appears in the cells which have not been treated with the FaPy-glycosylase enzyme. In the cells treated with FaPy-glycosylase the 350 and 500 J/m^2-treated cells have 8-OHdg sites at 0 hours post exposure to UVB, and the cells not treated with FaPy-glycosylase show no signs of damage. Therefore the damage measured in cells 0 hour post UVB exposure appears to be specifically 8-OHdg sites. However, the cells measured at 24 and 48 hours post exposure to UVB show the same level of DNA damage as the

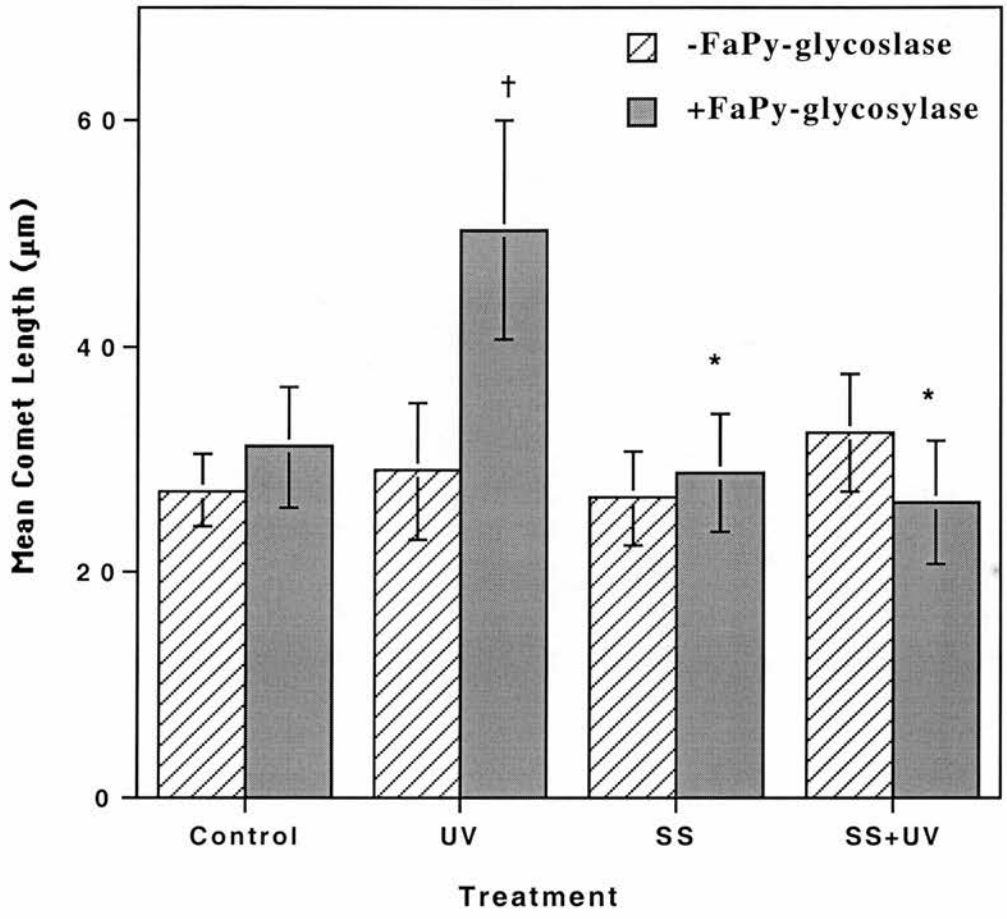
cells not treated with FaPy-glycosylase. Therefore damage measured at 24 and 48 hours post exposure to UVB, appears to be non-specific DNA damage.

Figure 5.12: Formation of 8-OHdg sites in primary keratinocytes exposed to UVB.



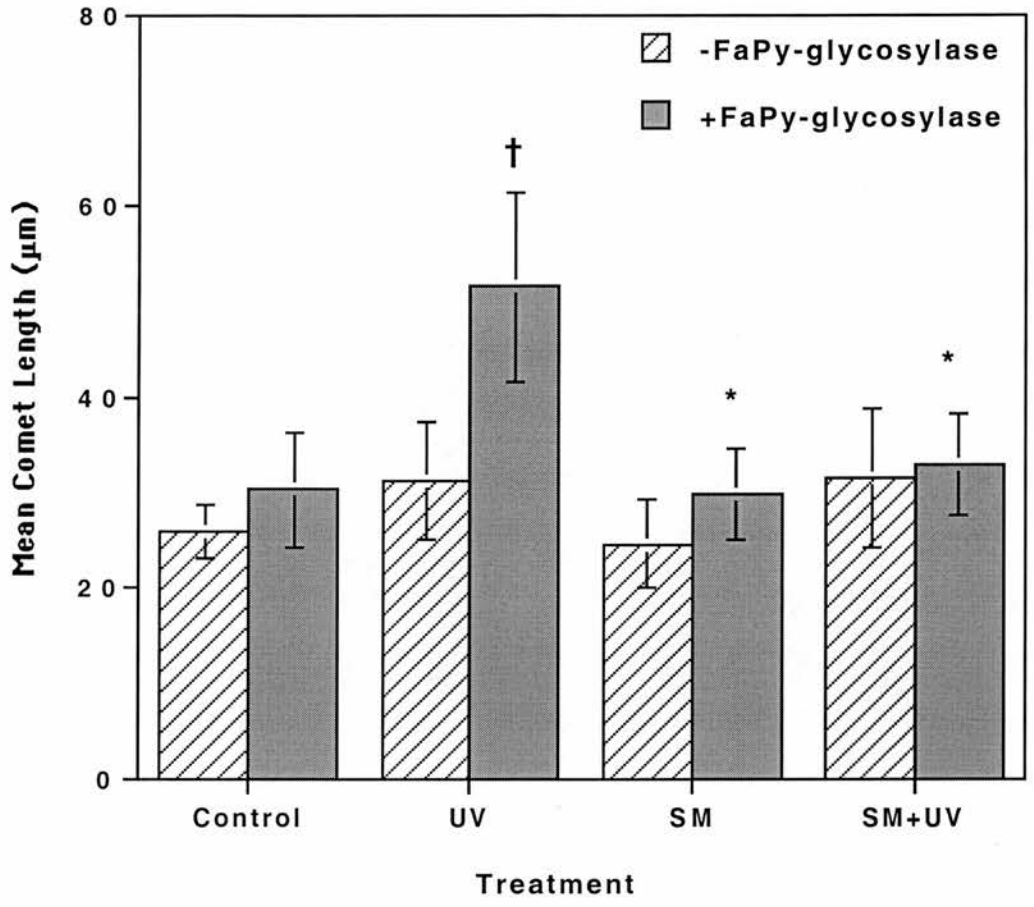
Primary keratinocytes were harvested and $2x10^4$ cells were mixed with low melting point agarose, layered onto slides and the cells exposed to filtered UVB (250-750 J/m^2). The cells were then incubated with FaPy enzyme prior to comet assay. The comets were scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment. Results are the mean comet lengths ± S.D., n = 100. * = significant increase in comet length, $p<0.05$.

Figure 5.13a: Effect of sodium selenite on the formation of 8-OHdg sites in primary keratinocytes exposed to UVB.



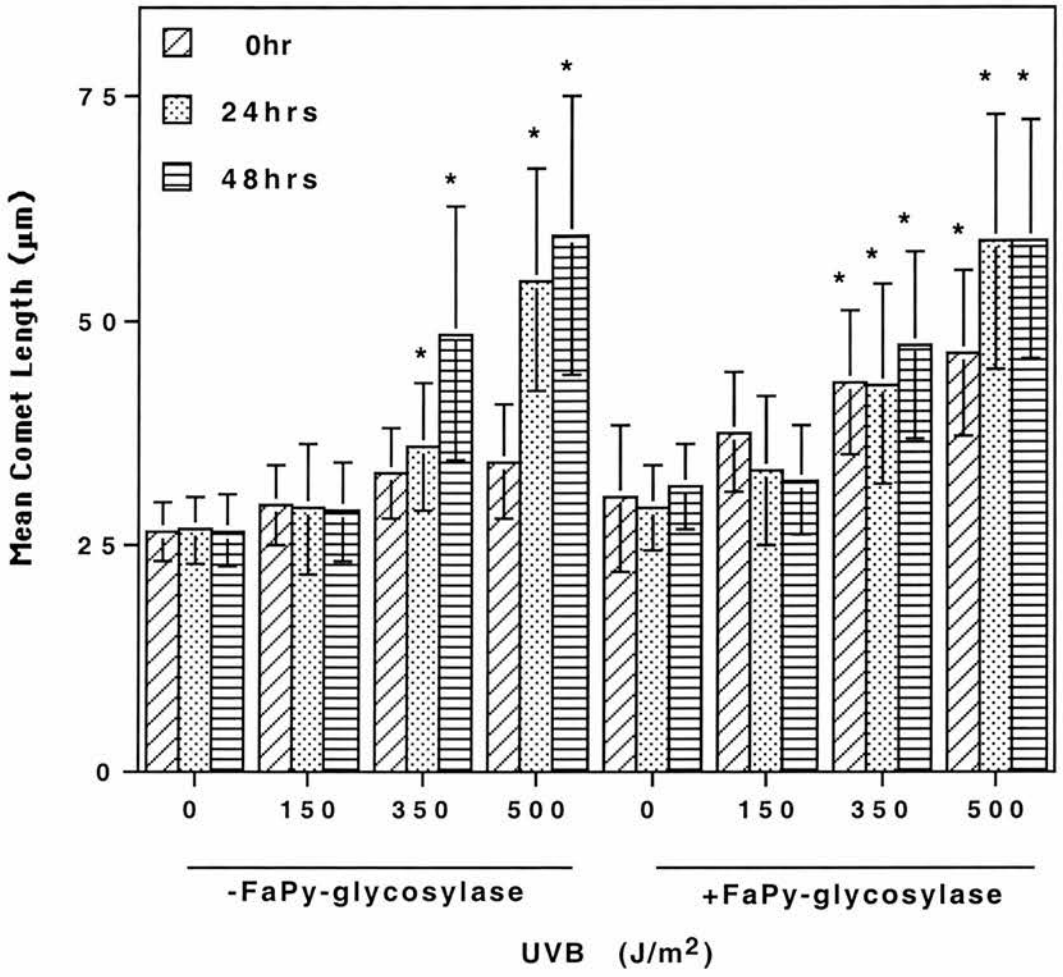
Primary keratinocytes were pre-treated with 50 nM sodium selenite (SS) for 24 hours prior to the cells being harvested, and $2x10^4$ cells were mixed with low melting point agarose and layered onto slides. The cells were exposed to filtered UVB (500 J/m^2) and then incubated with FaPy-glycosylase enzyme prior to the comet assay. The comets were scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment. Control cells have not been exposed to UVB or Se. Results are the mean comet lengths ± S.D., n = 100. † = significant increase in comet length, $p<0.05$. * = Significant decrease in comet length compared to irradiated, FaPy-glycosylase treated cells.

Figure 5.13b: Effect of selenomethionine on the formation of 8-OHdg sites in primary keratinocytes exposed to UVB.



Primary keratinocytes were pre-treated with 200 nM selenomethionine (SM) for 24 hours prior to the cells being harvested, and $2x10^4$ cells were mixed with low melting point agarose and layered onto slides. The cells were exposed to filtered UVB (500 J/m^2) and then incubated with FaPy-glycosylase enzyme prior to the comet assay. The comets were scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment. Results are the mean comet lengths ± S.D., n = 100. † = significant increase in comet length, $p<0.05$. * = Significant decrease in comet length compared to irradiated, FaPy-glycosylase treated cells.

Figure 5.14: Rate of repair 8-OHdg sites in primary keratinocytes exposed to UVB.



Primary keratinocytes were grown 3 cm^2 petri dishes and the media replaced with 1 ml PBS. The cells were then exposed to UVB (150-500 J/m^2), through the bottoms of the tissue culture dishes. The original media was then replaced and the cells were harvested at various time points following irradiation. Once harvested $2x10^4$ cells were mixed with low melting point agarose, layered onto slides and incubated with FaPy-glycosylase repair enzyme prior to the comet assay. The comets were then scored using an image analysis system, 50 cells were counted per slide with duplicate slides per treatment. Control cells have not been exposed to UVB or Se. Results are the mean comet lengths ± S.D., n = 100. * = significant increase in comet length, $p<0.05$.

5.3 Discussion.

Exposure of cells to all forms of UV radiation induces damage to DNA. Most of the DNA damage induced by exposure to UV radiation is repaired efficiently. However, if the damage burden is extensive then, the cells repair mechanisms may become overwhelmed and mutations within the DNA may be passed on to daughter cells. Failure to repair any damage to the DNA can lead to the initiation of carcinogenesis. If the mutated DNA alters the expression of a tumour suppressor gene, e.g. p53, or a gene which controls normal cell growth it can lead to unrestrained cell growth.

Exposure to UV radiation can lead to direct or indirect DNA damage. Direct DNA damage includes the formation of CPDs and 6-4 photoproducts. CPDs are the most common form of DNA injury, accounting for approximately 85% of the damage, with 6-4 photoproducts accounting for between 10-30% of observed damage induced by exposure to UVB. Direct forms of DNA damage are strongly linked to photocarcinogenesis. The analysis of basal and squamous cell carcinomas has revealed that the majority of mutations occur at dipyrimidine sites, resulting from direct DNA damage.

To measure the rate of repair of the DNA damage induced by exposure to UVB, DNA synthesis inhibitors were used in conjunction with the comet assay. The excision repair comet assay, allows the incision of the DNA at the site of damage. However the DNA synthesis inhibitors used in the assay, then prevent the resynthesis of new DNA to rejoin the DNA strands, therefore leading to an accumulation of single strand breaks in the DNA. The single strand breaks which accumulate can be specifically measured by the comet assay. When measuring DNA excision repair, a full set of controls were also run without the DNA synthesis inhibitors. Therefore any direct single strand breaks or any other DNA damage which is present can be detected. It was determined that the broadband UVB lamps used in this study do not induce many direct single or double strand breaks in DNA. It was also observed that no comets were detectable in any of the excision repair comet assay control slides (Fig 5.4 and 5.5).

It is thought that the direct forms of DNA damage detected, comprise the majority of damage induced by the low doses of UVB used in the excision

repair comet assay. Also CPDs and 6-4 products are the most abundant forms of DNA damage induced by exposure to UVB. The number of sites which were incised during the excision repair comet assay increased in a dose dependent manner with increasing levels of UVB (Fig 5.4). The low levels of UVB (5-20 J/m^2) required to induce DNA damage, which can be repaired by the excision repair mechanism, were in agreement with results reported by other authors. Doses of 1-50 J/m^2 UVB have been reported to induce direct DNA damage (Arlett *et al*, 1993; Collins *et al*, 1997). It should be noted that higher levels of exposure to UVB, will increase the level of DNA damage and consequently the number of single strand breaks. However, eventually so many strand breaks are present that the DNA forms smaller fragments. These smaller fragments of DNA can travel further away from the cell body, leading to the tail of the comet appearing detached from the cell body. Eventually the fragments are so small and travel so far that the resolution of the comet tail is lost. Therefore, the doses of UVB used in this study were selected in order that the levels of damage induced, formed comets with a continuous cell body and tail (Fig 5.10).

The results presented in Figure 5.5 display the effect the addition of Se, as sodium selenite or selenomethionine has on excision repair in primary keratinocytes. The addition of Se did not affect the degree of DNA damage which required excision repair. The lack of effect of the Se compounds was not totally unexpected, as the damage which is measured by the excision repair comet assay is mainly direct DNA damage. Therefore Se acting as an active component of antioxidant selenoproteins, would not affect the induction of direct DNA damage. However, it was thought possible that Se would perhaps speed up the process of excision repair, as Se is a component of the selenoprotein TR. Thioredoxin reductase is involved in the electron transfer chain for ribonucleotide reductase, an enzyme which reduces ribonucleosides directly to the corresponding deoxyribonucleoside. Ribonucleotide reductase requires thioredoxin for it's reducing power and thioredoxin is then recycled, using the selenoprotein thioredoxin reductase. Therefore, in conditions of low levels of Se, TR may be the rate limiting step in the production of new deoxyribonucleotides and hence new DNA. Therefore if excess Se was supplied to cells the optimal levels of TR would be expressed. Consequently, thioredoxin would be recycled more efficiently, ribonucleotide reductase would function more effectively, the pools of

deoxyribonucleotides would increase and so DNA repair would proceed more rapidly However, this was not observed and the process of excision repair was not altered by the addition of Se (Fig 5.5). The lack of effect of the increased levels of Se may be due to there already being sufficient levels of TR to reduce the thioredoxin present. The primary keratinocyte tissue culture media contains low levels of Se, this low level may be adequate to maintain sufficient levels of TR. Also in periods of Se deficiency, selenoproteins are conserved in some tissues, the selenoproteins which are conserved appear to be the essential forms. Therefore TR may be one of these conserved selenoproteins. The levels of TR present in the cells and the effect of Se supplementation on the levels of TR present in the cells, will be explored in Chapter 8. Finally it has also been established that other reductants can replace thioredoxin in the role of reducing ribonucleotide reductase, for example glutathione. Therefore even in conditions which are deficient in Se, the ribonucleotide reductase may use alternative reductants to thioredoxin.

The results concerning the formation and rate of repair of CPDs and the formation of DNA damage which requires excision repair are in close agreement. The similarity of results from the two comet assay methods, may be explained by the fact that CPDs are probably the major lesion which requires repair by the excision repair method. The CPDs were measured by incorporating a DNA repair enzyme into the comet assay method. In the case of CPDs it was T4 endonuclease V. The cells were irradiated, lysed, then washed with buffer to neutralise the lysis mix, in order that the DNA repair enzyme was not deactivated when added to the cells. The T4 endonuclease V was then added to the cells, were it selectively recognises the relevant DNA damage and cuts the DNA at the site of damage. However, because the cell has already been exposed to lysis buffer, it is no longer viable and the resynthesis of the damaged DNA can not occur. Therefore an increase in the number of single strand breaks in the DNA occurs.

The level of CPDs formed in the DNA increased in a dose-dependent manner and a dose of 5 J/m^2 was chosen to induce comets which were detectable (Fig 5.6). The dose of UVB used, was identical to the dose used to induced damage which required excision repair (5 J/m^2). It was also found

that Se did not affect the formation or repair of CPDs (Fig 5.7 and 5.8). The lack of effect of Se on the repair of CPDs, can probably be explained by the same reasons as the lack effect of Se on the formation and repair of excision repair damage. Firstly, because CPDs are a direct form of DNA damage thus ROS are not involved in their formation. Secondly, the levels of Se do not appear to altering the rate repair, via TR's actions on ribonucleotide reductase.

However, an interesting result was discovered when measuring the rate of repair of CPDs in primary keratinocytes. It was discovered that primary keratinocytes repair CPDs very quickly (Fig 5.9). It appeared that 50% of CPDs were repaired within 2 hours following exposure to UVB, by 3 hours post exposure only 5-10% of the CPDs remained and the remaining damage was repaired within 6 hours. Previously in the MRC Cell Mutation Unit in Brighton the rate of repair of CPDs in primary skin fibroblast had been shown to be relatively slow. Fibroblast do not appear to have repaired any of the CPDs present in their DNA by 6 hours post exposure to 5 J/m^2 UVB (Fig 5.11). Indeed it is only 24 hours post exposure to UVB, that the fibroblast have repaired approximately 50% of the CPDs and even 48 hours post exposure some damage remains. A longer time course was carried out by the MRC Cell Mutation Unit and some damage was still detected 72 hours following UVB exposure (Han *et al*, 1998). The rapid repair of CPDs in keratinocytes is an important observation as it suggests that, keratinocytes have selectively developed a repair pathway for the prompt repair of CPDs. A fast repair mechanism to deal with CPDs would be crucial for keratinocytes, as CPDs are the most common type of DNA damage induced by exposure to UVB radiation. Also keratinocytes are exposed to the highest levels of UVB, which is the wavelength of UV from the sun which induces the most CPDs (Kuluncsics *et al*, 1999). Fibroblasts however, are shielded from some of the harmful effects of UVB by overlying keratinocytes and melanocytes, therefore they are exposed to lower levels of UVB and appear less equipped to repair the damage which it induces.

Few studies have investigated the rate of repair of the major types of direct DNA damage in the skin. Also most of the investigations have been carried out using biopsy material, immunostaining and few time points. These studies do suggest that 6-4 photoproducts are repaired relatively quickly in

skin, with repair completed within 24 hours following exposure to UVB. The repair of 6-4 photoproducts has even been reported to be completed within 2 hours following exposure to UVB (McGregor, 1999). The quick repair of 6-4 photoproducts in the skin may suggest a similar rapid pathway of repair to that for CPDs.

There are conflicting reports in the literature concerning the rate of repair of CPDs. It has been reported that CPDs appear to be repaired more slowly than 6-4 photoproducts, with CPDs observed to remain in the skin 24-72 hours following exposure to UV radiation. The presence of CPDs were measured using immunostaining of skin biopsies from patients exposed to 2 MEDs of UVB (Young *et al*, 1996; Ahmed *et al*, 1999). In contrast, it has also been described that CPDs were repaired in human skin biopsies from patients exposed to UVB, within 4-12 hours following exposure (Freeman, 1988; Muramatsu *et al*, 1992; McGregor, 1999; Kinley *et al*, 1995).

There would appear to be large unresolved differences in the accepted rate of repair for CPDs in the epidermis, with times for completion of repair, following exposure to UVB ranging from 4-72 hours. The studies using human skin biopsies do not clarify, if the damage which remains for long periods of time is found in the basal keratinocytes, which are still replicating or is found higher in the epidermis. It may be that the slow rate of repair found in some of the studies is due to the damage being found in resting or differentiating keratinocytes. Therefore my study is the first to measure the rate of excision repair in primary keratinocytes *in vitro* where most of the keratinocytes population are basal replicating cells.

The final part of the my study investigated the formation and effect of Se on indirect DNA damage. Indirect DNA damage has also been reported to be involved in carcinogenesis (van der Scroeff *et al*, 1990; Nishigori *et al*, 1994) The formation of 8-OHdg was studied using another DNA repair enzyme called FaPy-glycosylase, which functions along the same principles as T4 endonuclease V. It however recognises 8-OHdg sites in the DNA.

Figure 5.12 indicates that increased levels of UVB are required to induce 8-OHdg sites in primary keratinocyte DNA (250-750 J/m^2), compared to the levels of radiation required to induce direct DNA damage (1-5 J/m^2). The

higher levels of UVB required to cause indirect DNA damage, indicate that the broadband UVB lamps used are less effective inducers of this form of DNA injury.

Selenium supplementation of primary keratinocytes decreased the formation of 8-OHdg sites in the DNA. Due to the limitations in the number of samples which could be processed, only one concentration of sodium selenite (50 nM) and one concentration of selenomethionine (200 nM) could be investigated. However both of the concentrations of Se investigated prevented the formation of 8-OHdg sites (Fig 5.13a and b).

Stewart *et al* have reported that pre-treatment with sodium selenite (5 μM) for 48 hours can reduce the formation of 8-OHdg sites in murine keratinocyte DNA following exposure to UVB (500 J/m^2) (Stewart *et al*, 1996). The study by Stewart *et al* corresponds well to the findings in the my study, the dose of UVB used was identical to that in the present study (500 J/m^2) and the induction of 8-OHdg was almost completely prevented with Se. However, the concentration of sodium selenite utilised was much higher (5 μM) than in the present study (50 nM). Indeed I have found that 5 μM sodium selenite supplementation, for 48 hours would have proven to be toxic to the keratinocytes (Chapter 3) and is non-physiological.

The 8-OHdg lesions appear to be produced as a consequence of the indirect production of H_2O_2 (reviewed in Thomas *et al*, 1998), hydroxyl radicals (Peak and Peak, 1990), singlet oxygen and lipid peroxidation (Park and Floyd, 1992) by UVB which then damage the DNA. Selenium may be protecting against the formation of 8-OHdg damage to DNA by acting via antioxidant selenoproteins. The increased levels of Se may increase the levels of GPX proteins or TR, thereby decreasing the levels of H_2O_2 and concomitantly decreasing the formation of the hydroxyl radical. The reduction in reactive species would then lead to less oxidative DNA damage. In addition increasing the levels of GPX and TR, would also lead to a decrease in the levels of lipid peroxidation, which would also diminish the levels of oxidative DNA damage. The effect of Se supplementation on the levels of lipid peroxidation induced by exposure to UVB have been investigated in Chapter 3. Supplementation of keratinocytes with either sodium selenite or selenomethionine decreased the formation of lipid

peroxides following exposure to UVB (1000 J/m^2). The decrease in lipid peroxidation may prove to be one of the factors involved in the decrease in the levels of 8-OHdg in keratinocyte DNA. TR can also act to decrease the levels of superoxide, which is also another causative factor in the formation of 8-OHdg damage.

The addition of other antioxidants to cells prior to their exposure to UV has been reported to decrease the formation of 8-OHdg damage. The antioxidants include; glutathione, which decreases the formation of 8-OHdg in DNA following exposure to ionising radiation (Fischer-Neilson *et al*, 1994). Ascorbic acid and vitamin E can also decrease the formation of 8-OHdg damage in keratinocytes exposed to UVB (Stewart *et al*, 1996).

The formation of other forms of oxidative DNA damage can be decreased by the addition of Se or other antioxidants. The other forms of oxidative damage which can be prevented include; single strand breaks induced by the exposure of fibroblasts to UVA, which can be decreased by the addition of N-acetylcysteine, sodium selenite (600 nM) or zinc (Emonet-Piccardi *et al*, 1998). Sodium selenite (50 nM) can also decrease the production of single strand breaks in DNA, induced in fibroblasts by exposure to H_2O_2 (Leist *et al*, 1996)

Finally the rate of repair of 8-OHdg sites in keratinocytes DNA was investigated. It was found however that when using 500 J/m^2 UVB, the level of damage increased over 48 hours. Also the level of damage found in the control slides increased overtime (Fig 5.13). Therefore, it appears that a chain reaction occurs and secondary or delayed DNA damage occurs in the cells, which is not specifically 8-OHdg lesions. It is thought that the damage which appears in the control cells is secondary oxidative damage or that exhibited by apoptotic cells. The formation of apoptotic cells can be measured using the comet assay. Cells undergoing the DNA cleavage step of apoptosis show comets. Indeed the dose of UVB used to cause 8-OHdg sites in the DNA, 500 J/m^2 UVB will induce significant apoptosis as illustrated in Chapter 4. Due to the irradiated non-enzyme treated cells becoming DNA damaged when using 500 J/m^2 UVB, lower doses of radiation were used in the time course experiment. However the cells exposed to 150 J/m^2 did not exhibit any 8-OHdg damage and when cells

were treated with 350 J/m^2 UVB, the control slides with no enzyme treatment showed signs of secondary DNA damage. Consequently a repair time course for 8-OHdg lesions could not be satisfactorily shown. However in the literature it is suggested that the rate of repair of 8-OHdg lesions is slow. It has also been reported that 8-OHdg lesions accumulate up to 24 hours following exposure to UVB and then begin to decline slowly from 48-96 hours post exposure to UV (Hattori-Nakakuki *et al*, 1994; Ahmed *et al*, 1999). Therefore it would appear that whilst 8-OHdg lesions are formed at a lower rate than direct DNA damage, they appear to exist in the DNA of keratinocytes for longer than CPDs and are produced at biologically significant levels of UVB.

5.4 Summary.

Selenium supplementation did not affect the formation or repair of direct forms of DNA damage. Selenium did however decrease the formation of indirect DNA damage, in the form of 8-OHdg. The full biological significance of indirect DNA damage is not known, however it has been linked to carcinogenesis. Also the 8-OHdg lesions appear to be repaired at a slower rate, than direct forms of DNA damage and so may be present in DNA for longer periods of time.

Finally it was also discovered for the first time *in vitro* that keratinocytes have a rapid method for the repair of CPDs. This is an important discovery considering that keratinocytes are exposed to the highest levels of UV radiation and CPDs are the most common lesion formed after exposure to UV radiation.

5.5 Further Work.

Further investigations need to be carried out on the effect of Se on the formation and rate of repair of 6-4 photoproducts, these are the second most common DNA lesion found following exposure to UVB. The levels of TR and GPX and the effect of Se supplementation, need to investigated in the epidermis. A greater concentration range of Se needs to be investigated when studying the formation 8-OHdg sites. The time for complete repair of

the 8-OHdg sites also needs to be followed. Finally investigations into which reactive species induce 8-OHdg sites, needs to be undertaken.

Chapter 6

Effect of Se on cytokine expression in primary human keratinocytes following exposure to UVB radiation.

6.1 Introduction.

The cytokine network is central to the immune system in the skin and to the recruitment of effector cells to the epidermis (the cytokines studied in this Chapter are summarised in Table 6.1). Many of the cytokines produced by the skin are thought to be involved in the sunburn reaction which occurs following exposure to sunlight, these include IL-8, IL-6 and IL-1 (reviewed in McKenzie and Sauder, 1994). Following exposure to UVB, oedema and swelling occur in the skin as an inflammatory infiltrate is attracted to the site. At higher doses of UV, systemic effects, such as fever can occur. The purpose of this inflammatory reaction following exposure to UV is unknown. Other cytokines are produced following exposure to UV light, which are responsible for the induction of immune suppression and include TNF-α , TGF-β and IL-10. Production of these cytokines may form part of a feed-back loop that helps to reduce the inflammatory reaction. However they can cause systemic immune suppression, thereby allowing highly immunogenic skin cancers to develop and evade the immune surveillance system.

6.1.1 Interleukin-6.

Interleukin-6 is an important mediator of B cell growth and differentiation, it enhances antibody production, functions as a secondary signal in T cell activation and induces fever and acute phase proteins (reviewed in Wong and Clark, 1988). Interleukin-6 can also increase keratinocyte proliferation (Grossman *et al*, 1989), and is an inflammatory cytokine which is thought to be involved in the sunburn reaction. The protein for IL-6 is released into the circulation following exposure to UVB light (Urbanski *et al*, 1990) and can be produced by fibroblast and keratinocytes (Kirnbauer *et al*, 1991; Wlaschek *et*

al, 1993). The peak period of IL-6 mRNA induction following exposure to UVB is at 6 hours, protein production peaks at 12 hours (de Vos *et al*, 1994).

6.1.2 Interleukin-8.

Interleukin-8 is a chemoattractant for neutrophils and T cells, and is produced by keratinocytes, dermal fibroblasts and endothelial cells (reviewed in Matsushima and Oppenheim, 1989). Interleukin-8 is a secondary cytokine and its expression is induced by primary cytokines such as IL-1 and TNF-α. IL-8 production can also be induced directly by the transcription factors; activator protein-1 (AP-1) (Mukaida *et al*, 1990) and nuclear factor kappa B (NFκB). Human keratinocytes produce IL-8, which is involved in the movement of T cells and neutrophils towards the epidermis (Barker *et al*, 1990). Inflammatory infiltrate (Hawk *et al*, 1988) and oedema have been demonstrated to be produced in rabbits and humans as a consequence of injection with IL-8 (Rampart *et al*, 1989; Leonard *et al*, 1991). Interleukin-8 production is also stimulated by exposure to UVB radiation, with the peak of mRNA production occurring at 6 hours post exposure and protein production at 24 hours post exposure (Kondo *et al*, 1993).

6.1.3 Tumour necrosis factor-α.

The pro-inflammatory cytokine TNF-α induces the secretion of IL-1 (Kutsch *et al*, 1993), IL-8 and IL-6 by keratinocytes. Furthermore, TNF-α also stimulates the expression of MHC class II, IL-2 receptor and adhesion molecules ICAM-2 and E-selectin on endothelial cells. The expression of endothelial adhesion molecules enables granulocytes, monocytes and lymphocytes to bind to the vessel walls and subsequently migrate into the tissues. TNF-α is implicated in the migration of LCs from the epidermis following exposure to UVB (Cumberbatch and Kimber, 1992; Yoshikawa *et al*, 1992). TNF-α has both AP-1 and NFκB binding sites in its promoter region (Spriggs *et al*, 1992). Moreover, TNF-α is also implicated in the induction of apoptosis following UVB exposure (Schwartz *et al*, 1995) and in the elicitation of contact hypersensitivity in the skin (Piguet *et al*, 1991). Although expressed at low levels in normal skin, TNF-α production by keratinocytes can be stimulated by exposure to UVB (Kock *et al*, 1990).

Induction of mRNA for TNF-α reaches its maximum between 6-12 hours, with protein production peaking at 24 hours following UVB exposure.

Kibitel *et al* have demonstrated that TNF-α induction by UVB is linked to DNA damage. Application of the DNA repair enzyme T4 endonuclease V reduces the production of TNF-α (Kibitel *et al*, 1998). It has been reported that antioxidants and NFκB inhibitors also reduce TNF-α production following UVB (Corsini *et al* 1995). This inhibition suggests that free radical induction of NFκB is a major inducer of TNF-α protein following UVB.

6.1.4 Interleukin-1α.

Interleukin-1α is a pro-inflammatory cytokine secreted by many cell types with a pivotal role in epidermal inflammation. It induces adhesion molecules on endothelial cells and stimulates the secretion of secondary cytokines such as IL-6 (Chung *et al*, 1996) and IL-8 (Larsen *et al*, 1989) from many cells, including fibroblasts and keratinocytes. Keratinocytes produce active IL-1α and the inactive IL-1β precursor, IL-1α also has an NFκB binding site within the promoter region. Unstimulated keratinocytes store large amounts of pre-formed IL-1α (Sauder *et al*, 1982), which is released upon damage to the epidermis (Kondo *et al*, 1994). The mRNA for IL-1α is only moderately increased at 2 hours post exposure to UVB and by 24 hours it is suppressed. However the pre-formed IL-1α protein is released from keratinocytes 6 hours after exposure to UVB (Kondo *et al*, 1994).

6.1.5 Interleukin-10.

Interleukin-10 is regarded as an inhibitory regulator of the immune system (Moore *et al*, 1993). It regulates the inflammatory immune response by inhibiting the synthesis of IL-1α, TNF-α, colony stimulating factor (CSF) (de Waal Malefyt *et al* 1992) and gamma interferon (γ-IFN) production by T cells and macrophages (Fiorentino *et al*, 1991; Enk, 1994). In the skin IL-10 is induced in contact sensitivity responses in mice (Enk and Katz, 1992) and may have a role in limiting the inflammatory response induced by this treatment. Furthermore IL-10 can inhibit the ability of LCs to elicit a delayed type hypersensitivity response to tumour associated antigens (Beissert *et al*, 1995). Exposure of murine keratinocytes *in vitro* to UVB induces IL-10

expression (Rivas and Ullrich, 1992). Interleukin-10 has also been shown to reduce the production of inducible nitric oxide synthetase (iNOS), nitric oxide, TNF-α and IL-6 by primary human keratinocytes (Becherel *et al*, 1995). However the production of IL-10 in human keratinocytes *in vitro* remains controversial since some investigators have identified IL-10 protein and mRNA in UVB exposed human keratinocytes (Grewe *et al*, 1995; Enk *et al*, 1995), whilst others could not (Teunissen *et al*, 1997). Teunissen has suggested that the IL-10 found in human skin originates from melanocytes (Teunissen *et al*, 1997, Mattei *et al*, 1994). Melanomas have also been shown to produce IL-10 and it is thought that this is part of the mechanism by which they evade the body's immune response (Kruger-Krasagakes *et al*, 1994). Exposure to UVB stimulates IL-10 mRNA transcription in mouse keratinocytes 8 hours after irradiation and protein production begins after 24 hours (Enk *et al*, 1995).

It has also been demonstrated that IL-10 production by mouse keratinocytes is induced by cyclopyrimidine dimers, caused by UVB irradiation (Nishigori *et al*, 1996). When the DNA repair enzyme T4 endonuclease V is applied to cells following exposure to UVB radiation it helps to repair the cyclobutane dimers quickly, which leads to a decrease in the production of IL-10. Consequently there is a reduction in the immune suppressive effects of IL-10. Recently further work has been carried out, which demonstrates that the application of T4 endonuclease V containing liposomes to human skin can prevent the UVB-induction of IL-10 and TNF-α, but not erythema (Wolf *et al*, 2000). However DNA damage is only one of the mechanisms by which cytokines are induced in the skin (O'Connor *et al*, 1996).

6.1.6 Cytokines and Se.

There is some evidence that implies Se can affect the levels of cytokines in the skin. When inflamed and normal skin are exposed in culture to 800 nM selenium chloride, sodium selenate or sodium selenite for 2 weeks, IL-1α, TNF-α and IL-6 protein levels were decreased in inflamed skin by the sodium selenite and selenate. The decrease in cytokine concentrations were detected by ELISA and immunostaining (Celerier *et al*, 1995). Selenium can also affect many other aspects of the immune system, as covered in Chapter 1 (reviewed in McKenzie *et al*, 1998). Se supplied both topically or in the diet

can reduce the inflammatory response following UV exposure in humans (Overvad *et al*, 1985; Burke *et al*, 1992a; Burke *et al*, 1992b) and in mice (Thorling *et al*, 1983). Following exposure to UVB there are two possible mechanisms by which Se may decrease the inflammatory response; by decreasing the release of inflammatory cytokines or by increasing the release of immune suppressive cytokines.

6.1.7 Transcription factor activation and Se.

There is a large body of literature to suggest that transcription factors can be upregulated by free-radicals and that Se can prevent this upregulation. NFκB is found in the majority of cell types, and is described in detail in Chapter 1. NFκB can increase IL-6 mRNA production in keratinocytes and is itself activated by exposure to UVB light and by free radicals (reviewed in Flohe *et al*, 1997). Free radical scavengers such as N-acetylcysteine, pyrrolidine-dithiocarbamate and thioredoxin (Schenk *et al*, 1994) decrease NFκB activation by UVB. This decrease in NFκB induction suggests that free radicals are involved in the induction of NFκB by UVB (Simon *et al*, 1994). Overexpression of PHGPX (by transfection of the DNA) in cells supplemented with 50 nM sodium selenite diminishes the activation of NFκB by IL-1; and selenite treatment alone for 4 days can also decrease the level of DNA binding by NFκB (Brigelius-Flohe *et al*, 1997). This implies that H_2O_2 or lipid peroxides are responsible for the activation of NFκB, because these are the target of PHGPX.

Another transcription factor which is found in most nucleated cell types is AP-1, it is also fully described in full in Chapter 1. AP-1 is also regulated by free radicals as the redox state of the components affects how well the complex binds to DNA (reviewed in Palmer and Paulson, 1997; Roebuck *et al*, 1999). AP-1 is also activated by UV light (Derijard *et al*, 1994; Devary *et al*, 1991). Again antioxidants such as N-acetylcysteine and glutathione can decrease the activation of AP-1 (Devary *et al*, 1991). Selenite and selenodigluthione added to nuclear extracts can inhibit AP-1 DNA binding, however high concentrations of Se, 7.5 μM and 750 nM respectively were required (Spyrou *et al*, 1995). The decrease in AP-1 binding was also confirmed in lymphocytes were cells were incubated with 10 μM selenite for 4 hours and a reduction in DNA binding was shown. The ability of selenite

to decrease the activation of AP-1 may help to explain the growth inhibitory effects of selenite at high concentrations.

Table 6.1 : Cytokines studied in this chapter.

Cytokine	Main function	UVB -induction mRNA	UVB -induction protein
IL-6	Inflammatory	6 hrs	12 hrs
IL-8	Inflammatory	6 hrs	24 hrs
TNF-α	Inflammatory, also involved in LC migration	6-12 hrs	24 hrs
IL-1α	Inflammatory	2 hrs	6 hrs
IL-10	Immune suppressive	8 hrs	24 hrs

The aims of the experiments described in this chapter were-:

- To confirm that UVB induces an increase in the levels of mRNA and protein for IL-6, IL-8, TNF-α, IL-10 and IL-1α in keratinocytes.

- To ascertain if Se modifies the induction of mRNA for the above cytokines following exposure to UVB.

- To determine if Se alters the release of IL-6, IL-8, IL-10 and TNF-α protein following exposure to UVB.

6.2 Results and Methods.

6.2.1 Semi Quantitative RT-PCR to measure levels of cytokine mRNA.

6.2.1.1 Optimum cycle number determination.

In order to analyse mRNA expression semi-quantitatively, the optimum cycle number for each PCR product was determined. A pool of cDNA was taken from the primary keratinocyte experiments or the Pam 212 cells (spontaneously transformed mouse keratinocyte cell line) experiments and were used as the targets for the cycle experiments. Figure 6.1, 6.2, 6.3 and 6.4 demonstrate the cycle numbers used. Forty cycles was the maximum cycle number used in this study, since above this number the reaction is likely to plateau due to exhaustion of the DNA polymerase and other reagents. The full RNA extraction method, RT-PCR and SDS-PAGE electrophoresis method are described in Chapter 2.

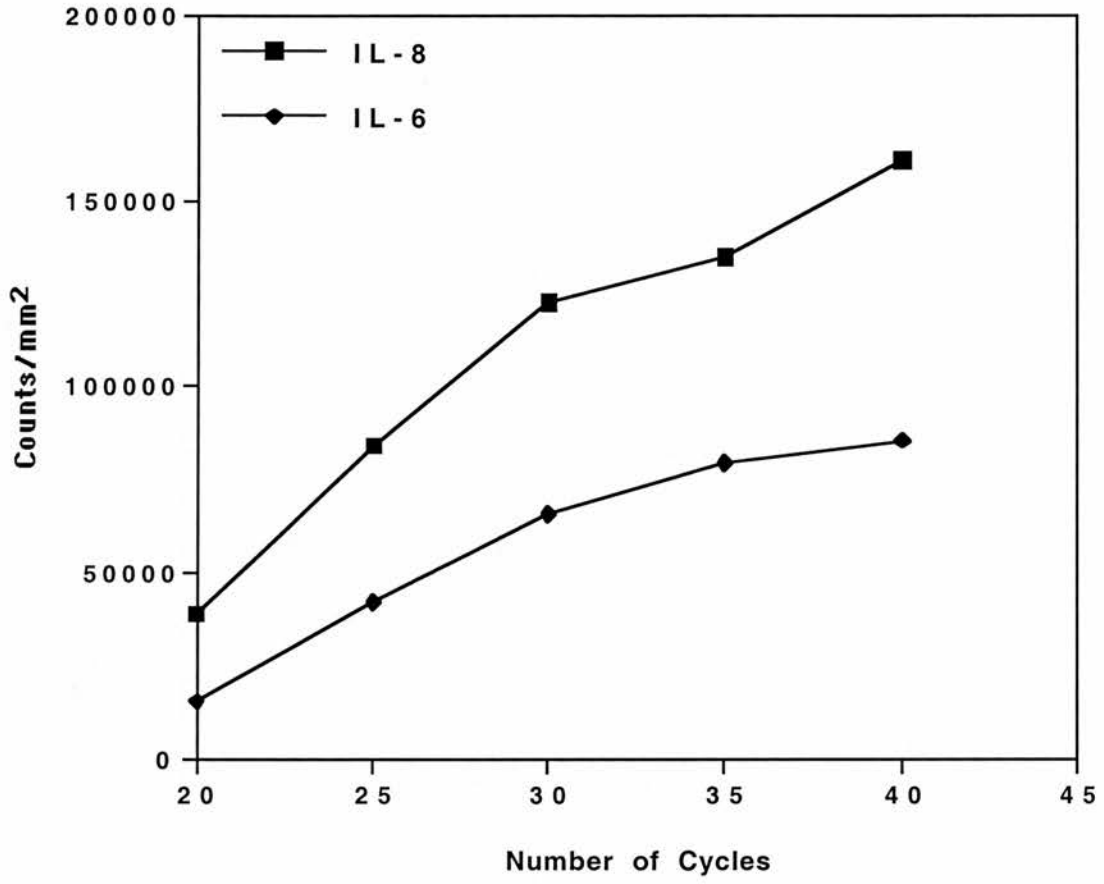
Optimum cycle numbers for each cytokine mRNA were determined. The optimum cycle numbers for the primary keratinocyte experiments for IL-6 and IL-8 were found to be 32 cycles (Fig 6.1). The optimum cycle number for TNF-α was 37 cycles, for IL-1α 35 cycles and for G3pDH 40 cycles (Fig 6.2). For the Pam 212 cell experiments the optimum cycle numbers were; for β-actin 32 cycles (Fig 6.3a and b), TNF-α 36 cycles and IL-10 40 cycles (Fig 6.4). These cycle numbers were used for the rest of the mRNA experiments.

6.2.1.2 Effect of UVB on cytokine mRNA levels in primary keratinocytes.

The effect of UVB exposure on primary human keratinocytes *in vitro* was examined. Time-course experiments were carried out on primary keratinocytes. The cells were exposed to UVB (200 J/m^2) then incubated for 3-24, hours, the cells were harvested, the RNA extracted and RT-PCR performed for the cytokines of interest. The house keeping gene G3pDH was used to normalise the levels of cDNA present. The dose of UVB was chosen as it has frequently been described in the literature to induce the

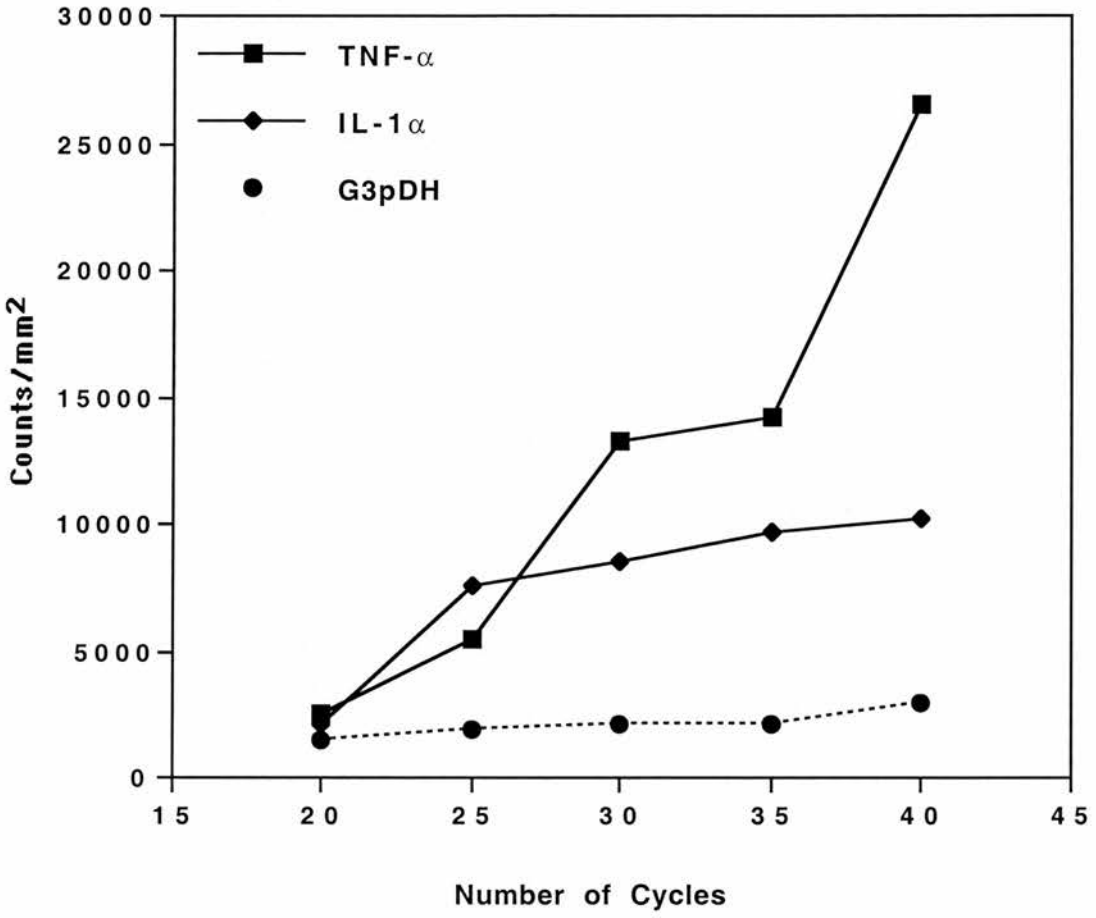
Continued on page 6-13

Figure 6.1: Effect of increasing cycle number on the amount of PCR product formed for the cytokines IL-6 and IL-8.



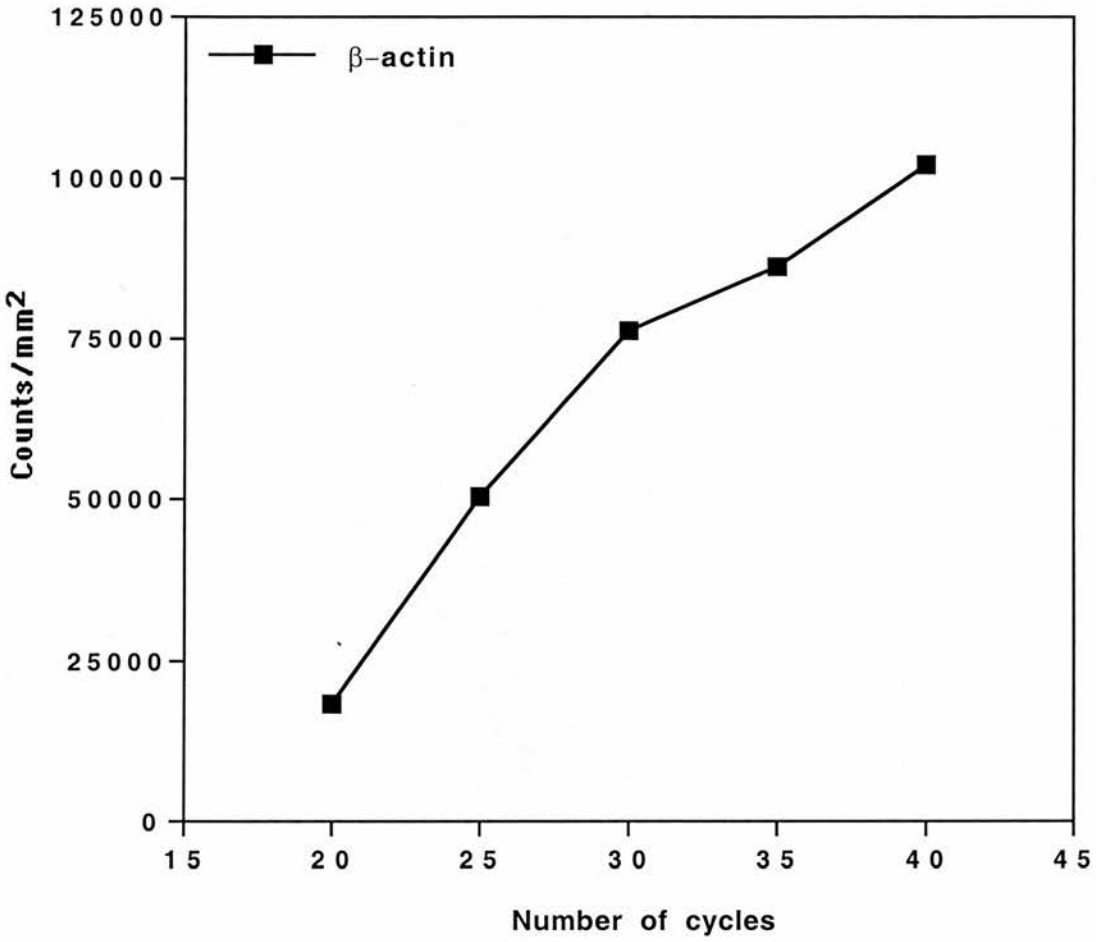
Primary keratinocyte cDNA from the experiments carried out in sections 6.2.1.2 and 6.2.1.3 were pooled and PCR reactions with ^{32}P-labelled primers were carried out with increasing cycle numbers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. Results are expressed as counts/mm^2.

Figure 6.2: Effect of increasing cycle number on the amount of PCR product formed for the cytokines TNF-α, IL-1α and G3pDH.



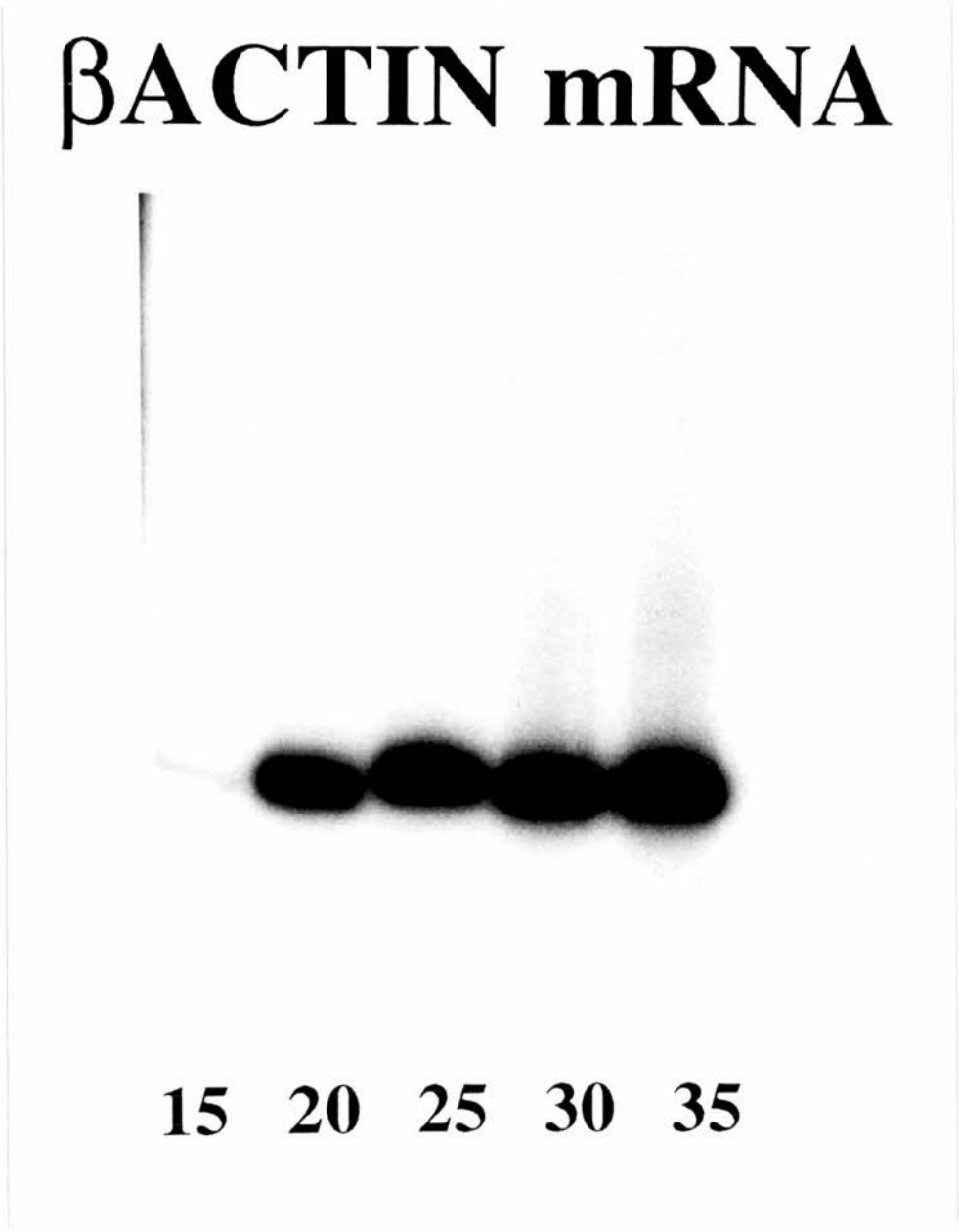
Primary keratinocyte cDNA from the experiments carried out in sections 6.2.1.2 and 6.2.1.3 were pooled and PCR reactions with ^{32}P-labelled primers were carried out with increasing cycle numbers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. Results are expressed as counts/mm^2.

Figure 6.3a: Effect of increasing cycle number on the amount of PCR product formed for β-actin.



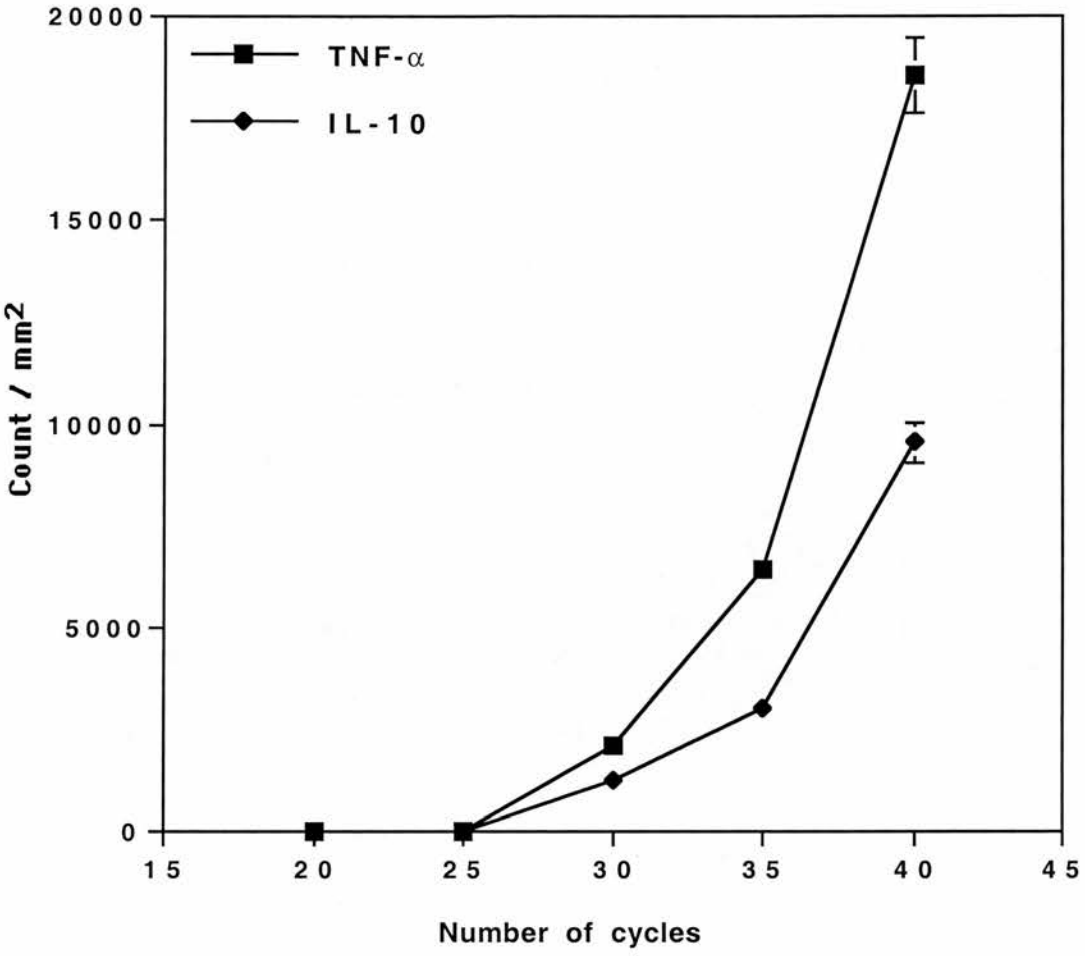
Pam 212 cells cDNA from the experiments carried out in section 6.2.1.5 were pooled and PCR reactions with ^{32}P-labelled primers were carried out with increasing cycle numbers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. Results are expressed as counts/mm^2.

Figure 6.3b: Effect of increasing cycle number on the amount of PCR product formed for β-actin.



Pam 212 cells cDNA from the experiments carried out in sections 6.2.1.5 were pooled and PCR reactions with ^{32}P-labelled primers were carried out with increasing cycle numbers. The PCR products were resolved on SDS-PAGE gels and exposed to autoradiography.

Figure 6.4: Effect of increasing cycle number on the amount of PCR product formed for the cytokines TNF-α and IL-10.



Pam 212 cells cDNA from the experiments carried out in sections 6.2.1.5 were pooled and PCR reactions with ^{32}P-labelled primers were carried out with increasing cycle numbers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. Results are expressed as counts/mm^2.

release of cytokines in normal human keratinocytes (De Vos *et al*, 1994; Kondo *et al*, 1994; Kondo *et al*, 1993) also at this dose there was no loss of viability of the cells (Chapter 3, Fig 3.2a). The time course experiments were performed in order that the optimum time point for the induction of each cytokine mRNA, following exposure to UVB could be discovered. This could then be utilised in further experiments.

Induction of IL-6 mRNA peaked at 6 hours post exposure to UVB, then declined slowly but was still slightly elevated 24 hours after exposure (Fig 6.5). The mRNA for IL-8 increased at 6 hours post exposure to UVB, then decreased slowly but was still elevated 24 hours post exposure to UVB (Fig 6.6). The TNF-α mRNA increased at 6 hours but decreased more rapidly over the 24 hours following exposure to UVB (Fig 6.7). The induction at 6 hours was found not to be significant due to large variation in the replicate samples. However significant induction of the mRNA has been demonstrated with UVB in the literature (Kock *et al*, 1990) Finally IL-1α mRNA was slightly increased at 6 hours and this had decreased by 12 hours post exposure to UVB (Fig 6.8). However the increase at 6 hours was not found to be significant due to the very small increase detected.

6.2.1.3 Effect of Se on the basal levels of mRNA for IL-6, IL-8, TNF-α and IL-1α. in primary keratinocytes.

Primary human keratinocytes were exposed to various concentrations of sodium selenite or selenomethionine for 24 hours, then the mRNA was extracted and RT-PCR performed on the samples. The house keeping gene G3pDH was again used to normalise the levels of cDNA present.

Basal levels of IL-6 mRNA were decreased significantly by 50 nM selenomethionine only (Fig 6.9). The basal levels of IL-1α mRNA were decreased at only one dose of selenite (1 nM) but not by selenomethionine (Fig 6.9). In contrast basal mRNA levels for IL-8 were decreased significantly at concentrations of selenomethionine ranging from 50-200nM and sodium selenite ranging from 1 nM to 10 nM (Fig 6.9). Finally the basal levels of TNF-α were not decreased significantly at any of the levels of

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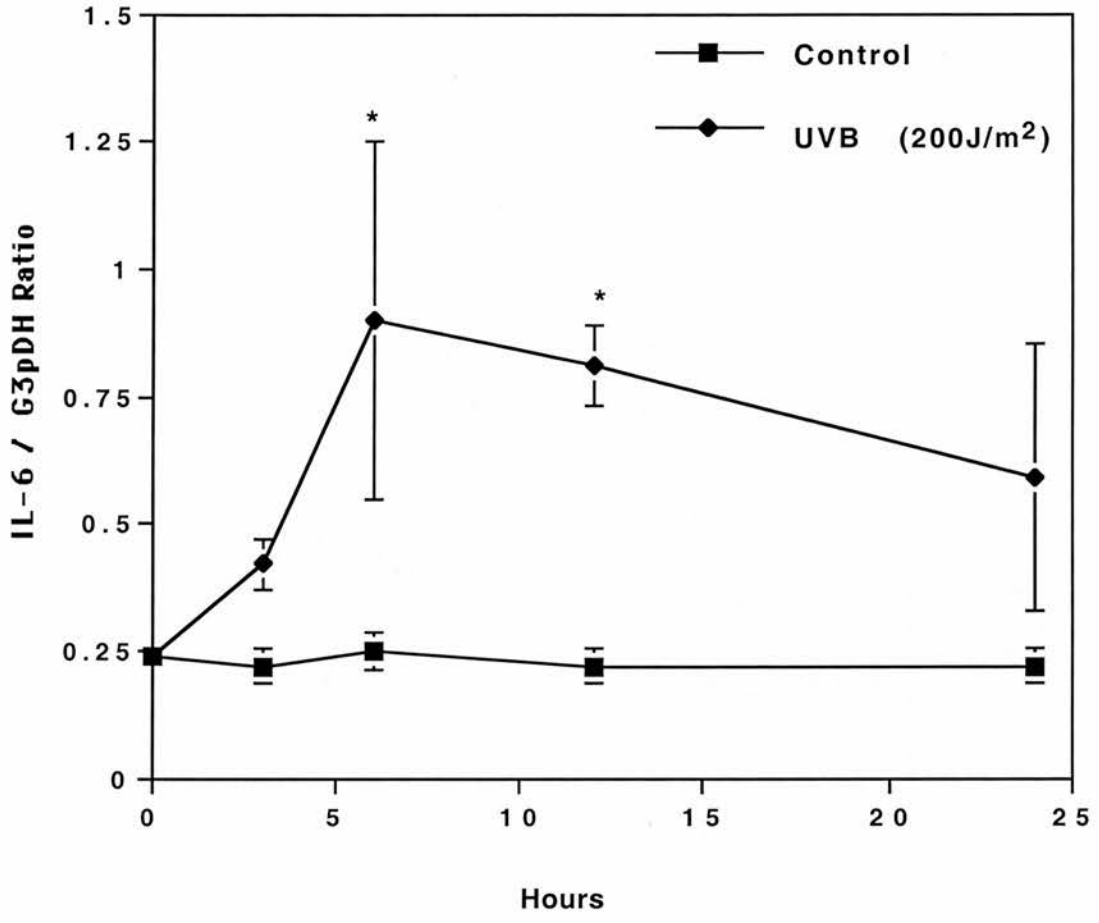
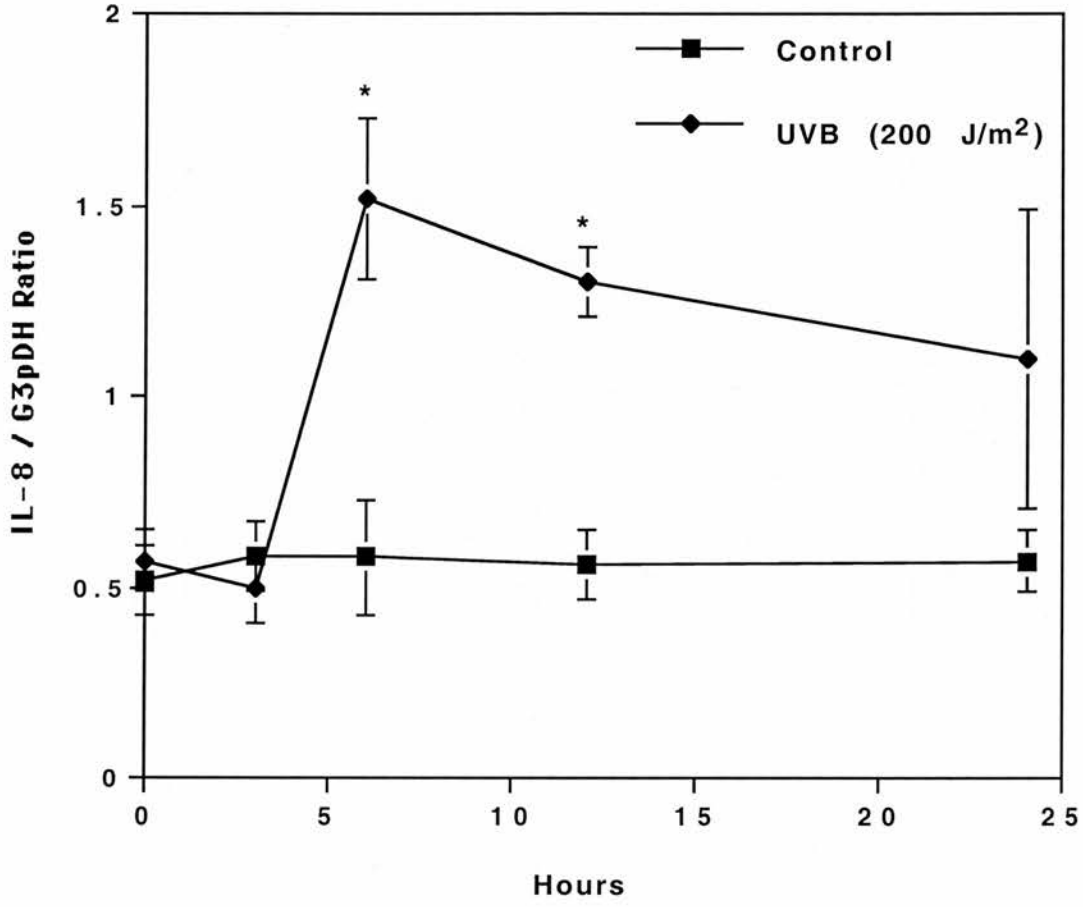
Figure 6.5: Levels of IL-6 mRNA in primary keratinocytes following exposure to UVB.



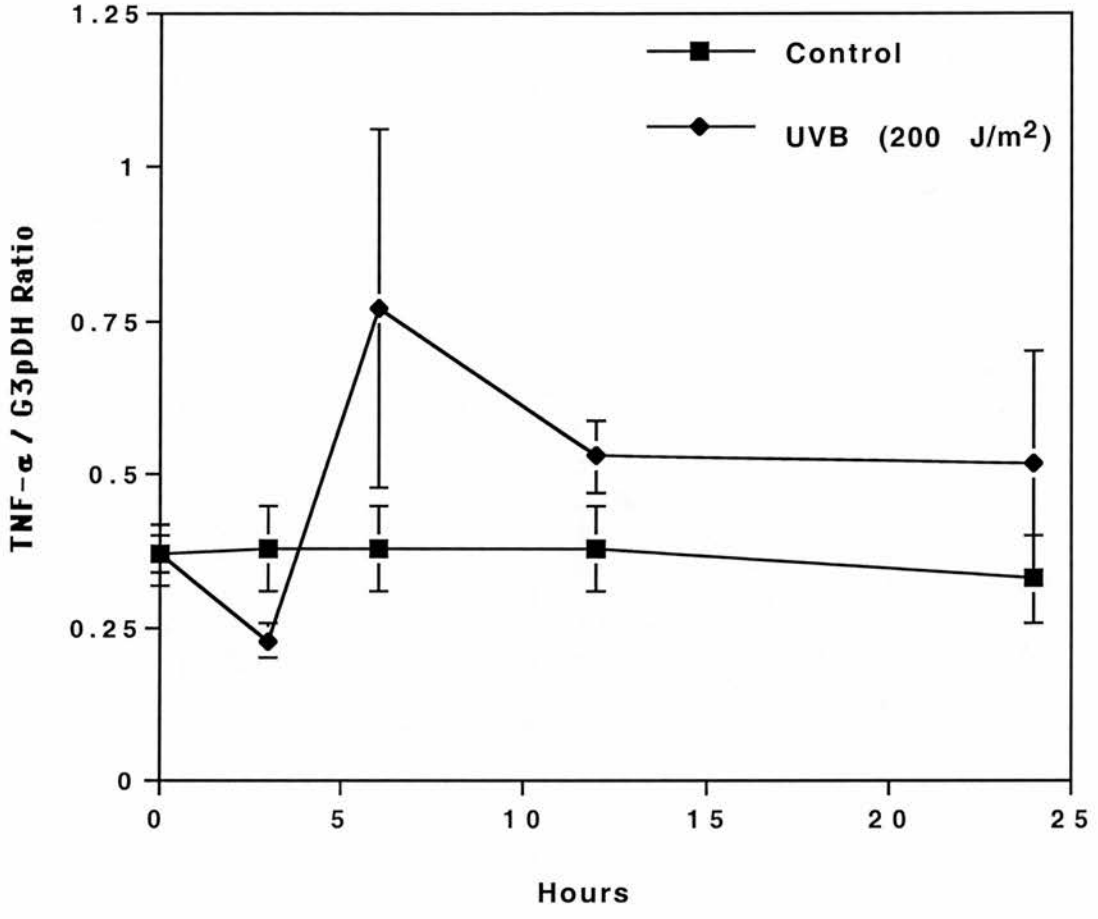
Primary keratinocytes were either exposed to UVB (200 J/m^2) or mock irradiated (control), the cells were incubated for 3-24 hours, prior to being harvested and the RNA extracted. RT-PCR was then carried out on the samples using ^{32}P-labelled primers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. The cytokine levels were normalised using the housekeeping gene G3pDH. Results are expressed as the mean ratios ± S.E.M, n=3. Significant difference from the control cells, * =$P<0.05$. The experiment was carried out with triplicate samples, two times.

Figure 6.6: Levels of IL-8 mRNA in primary keratinocytes following exposure to UVB.



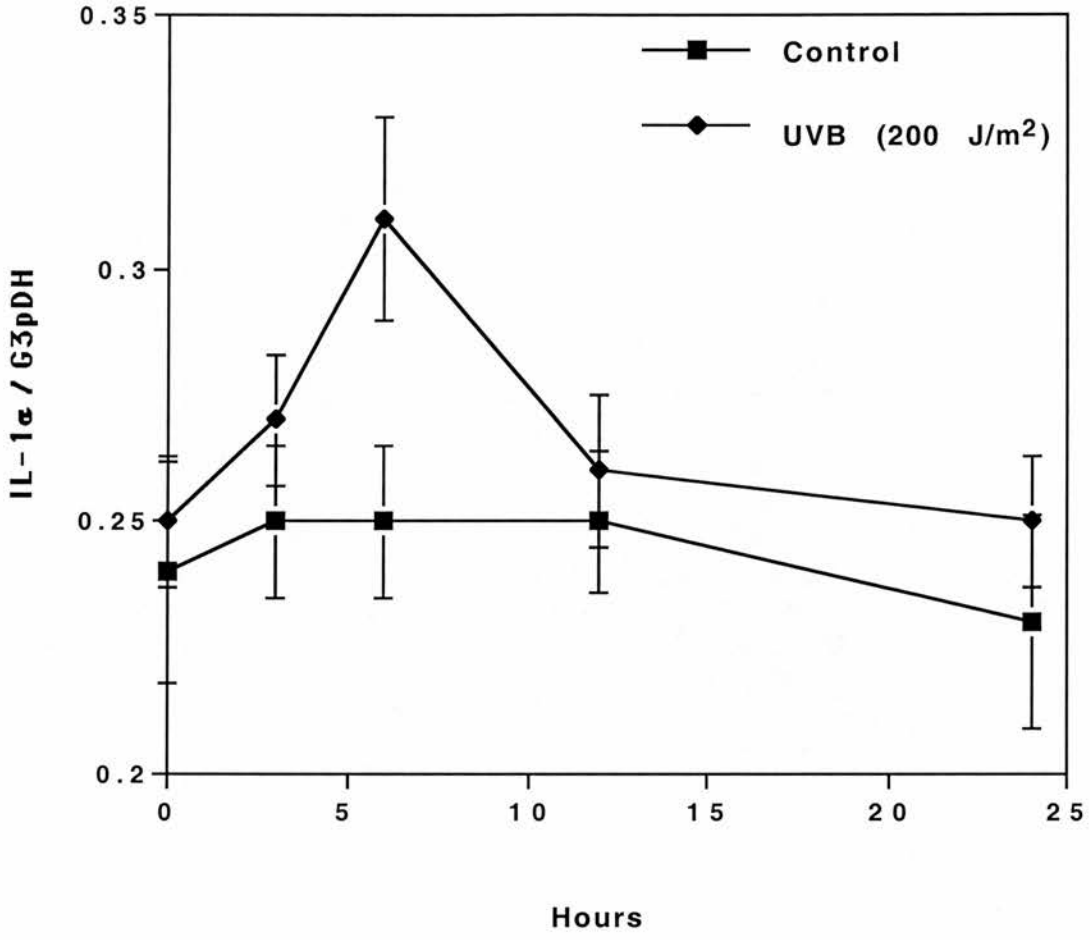
Primary keratinocytes were either exposed to UVB (200 J/m^2) or mock irradiated (control), the cells were incubated for 3-24 hours, prior to being harvested and the RNA extracted. RT-PCR was then carried out on the samples using ^{32}P-labelled primers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. The cytokine levels were normalised using the housekeeping gene G3pDH. Results are expressed as the mean ratios ± S.E.M, n=3. Significant difference from the control cells, * =$P<0.05$. The experiment was carried out with triplicate samples, two times.

Figure 6.7: Levels of TNF-α mRNA in primary keratinocytes following exposure to UVB.



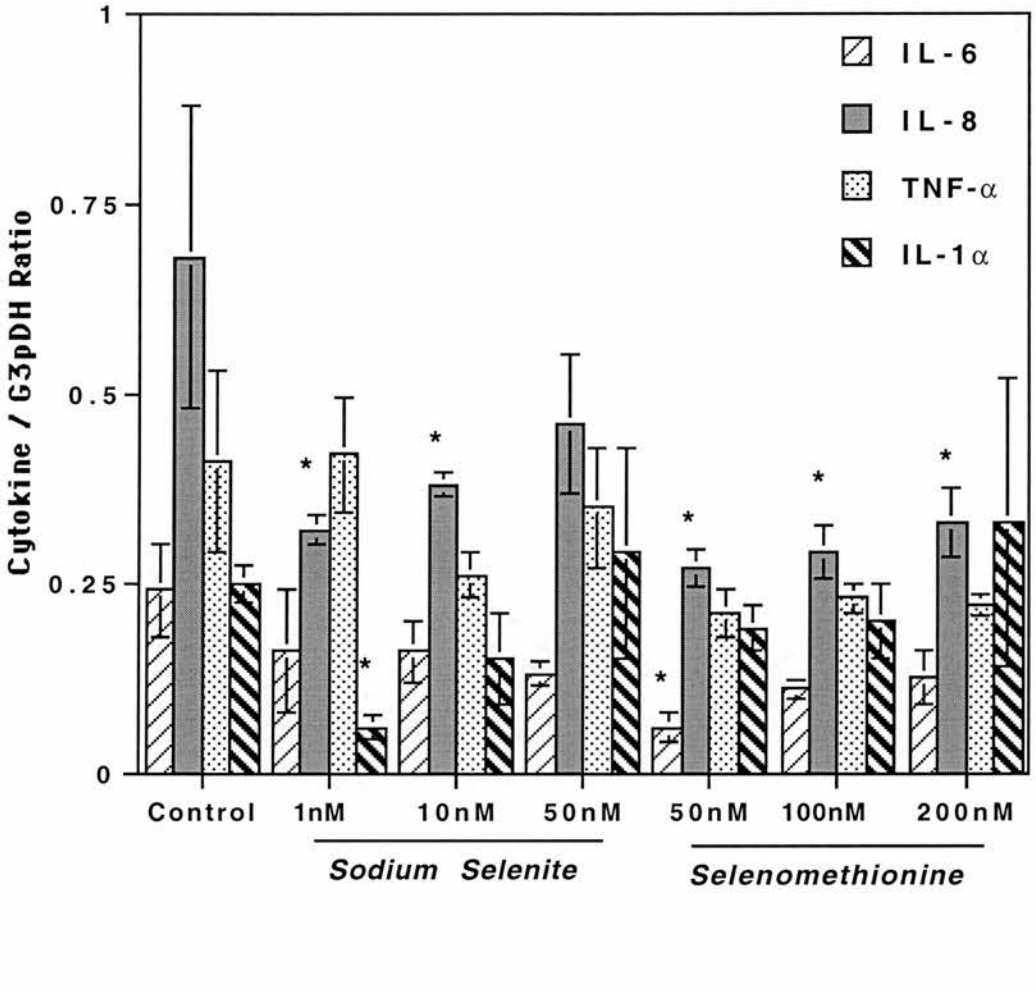
Primary keratinocytes were either exposed to UVB (200 J/m^2) or mock irradiated (control), the cells were incubated for 3-24 hours, prior to being harvested and the RNA extracted. RT-PCR was then carried out on the samples using ^{32}P-labelled primers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. The cytokine levels were normalised using the housekeeping gene G3pDH. Results are expressed as the mean ratios ± S.E.M, n=3. The experiment was carried out with triplicate samples, two times.

Figure 6.8: Levels of IL-1α mRNA in primary keratinocytes following exposure to UVB.



Primary keratinocytes were either exposed to UVB (200 J/m^2) or mock irradiated (control), the cells were incubated for 3-24 hours, prior to being harvested and the RNA extracted. RT-PCR was then carried out on the samples using ^{32}P-labelled primers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. The cytokine levels were normalised using the housekeeping gene G3pDH. Results are expressed as the mean ratios ± S.E.M, n=3. The experiment was carried out with triplicate samples, two times.

Figure 6.9: Effect of Se on the basal levels of mRNA for IL-6, IL-8, TNF-α and IL-1α in primary keratinocytes.



Primary keratinocytes were supplemented with either sodium selenite or selenomethionine for 24 hours prior to cells being harvested and RNA extracted. RT-PCR was carried out the samples using ^{32}P-labelled primers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. The cytokine levels were normalised using the housekeeping gene G3pDH. Control cells had no Se added. Results are expressed as the mean ratios ± S.E.M, n=3. Significant difference from the control cells, * =$P<0.05$. The experiment was carried out with triplicate samples, two times.

sodium selenite or selenomethionine tested (Fig 6.9).

6.2.1.4 Effect of Se on the UVB induction of mRNA for IL-6, IL-8, TNF-α and IL-1α by primary keratinocytes.

The effect of Se on the UVB induction of cytokines by keratinocytes *in vitro* was examined. The cells were supplemented with different concentrations of Se for 24 hours then exposed to UVB (200 J/m^2). After a further incubation of 6 hours, the cells were harvested and RNA extracted, subsequently RT-PCR was performed to quantify the mRNA for the cytokines of interest. The RT-PCR reactions were done using the cycle numbers described in section 6.2.1.1 and at the time points obtained in section 6.2.1.2.

Selenium supplementation with either selenite (1 nM) or selenomethionine (50-200 nM) prevented the induction of IL-6 mRNA by UVB which was observed in the absence of Se. Concentrations of selenite as low as 1 nM were sufficient to prevent induction of IL-6 mRNA (Fig 6.10a and b).

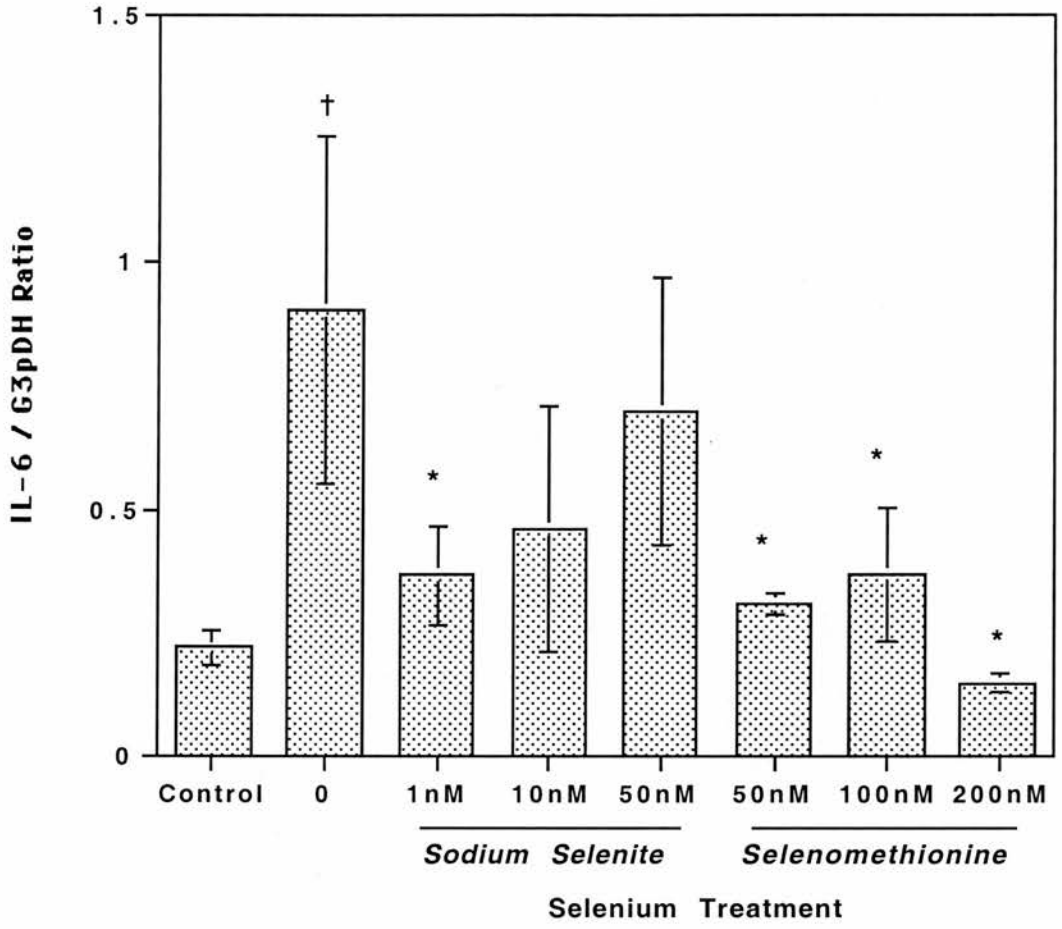
There was significant UVB induction of the IL-8 mRNA at 6 hours post exposure to UVB, Se reduced this induction at concentrations between 1-50 nM sodium selenite and at 50 and 200 nM selenomethionine (Fig 6.11a). Figure 6.11b presents the autoradiographs for IL-8 mRNA and the house keeping gene G3pDH.

The induction of TNF-α mRNA by UVB was not significantly different compared to control samples. However it can be seen from Fig 6.12 that the mRNA levels were significantly lower in irradiated cells treated with 100 nM and 200 nM selenomethionine, compared to cells which were irradiated but not pre-treated with Se.

There was no significant induction of IL-1α at 6 hours and no significant reduction of IL-1α mRNA at any of the concentrations of Se (Fig 6.13).

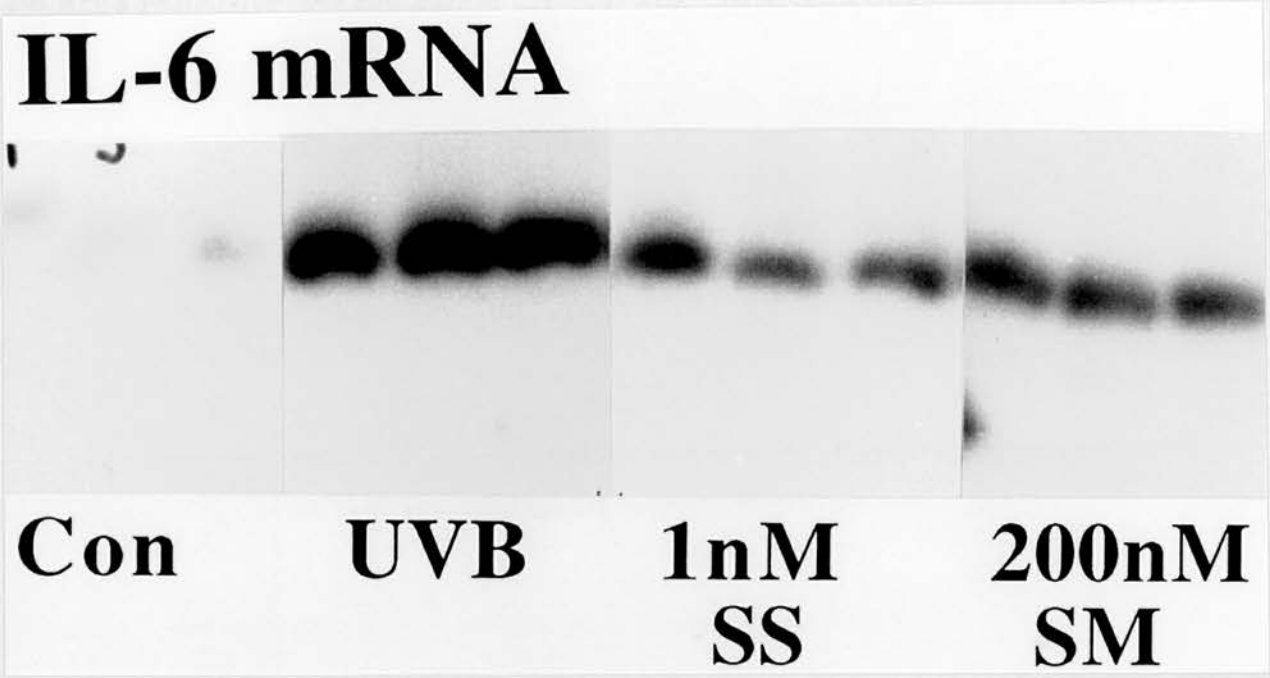
Continued on page 6-26

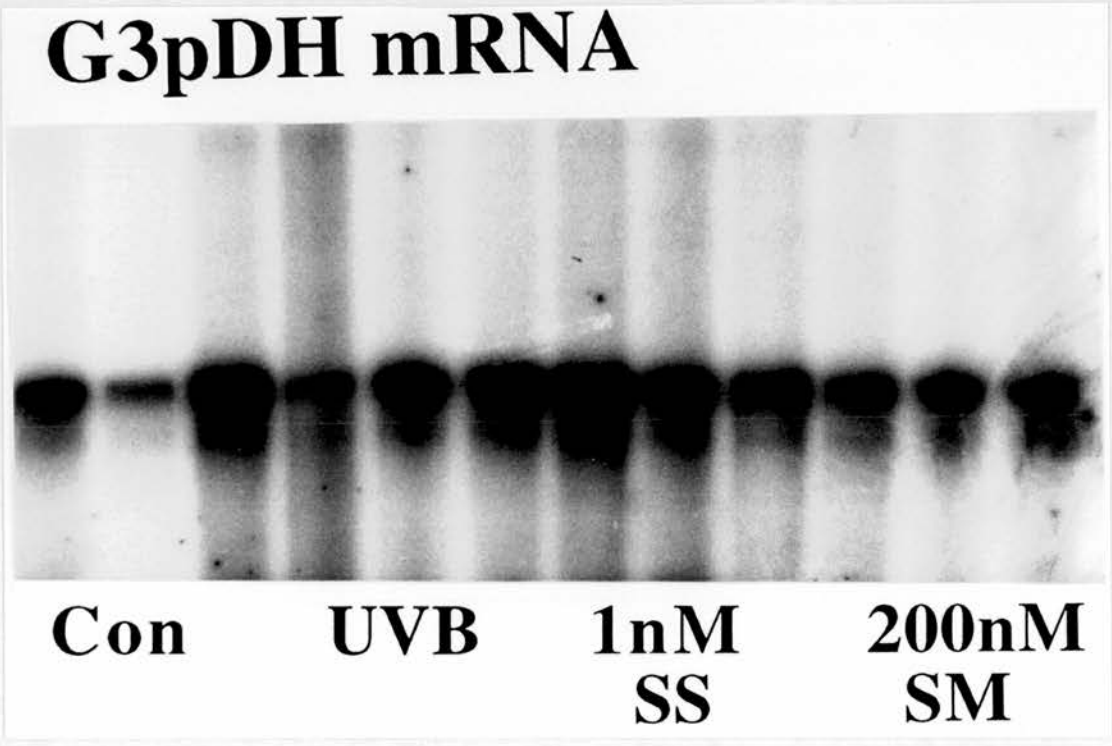
Figure 6.10a: Effect of Se on the levels of IL-6 mRNA, following exposure to UVB.



Primary keratinocytes were supplemented with either sodium selenite or selenomethionine for 24 hours prior to cells being exposed to UVB (200 J/m^2). Fresh media was returned to the cells and they were incubated for 6 hours before being harvested. The RNA was extracted and RT-PCR analysis was carried out on the samples using ^{32}P-labelled primers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. The cytokine levels were normalised using the housekeeping gene G3pDH. Control cells had no Se added and were mock irradiated. Results are expressed as the mean ratios ± S.E.M, n=3. Significant difference from the control cells, † = $P<0.05$, significant difference from the irradiated cells with no Se added * = $P<0.05$.

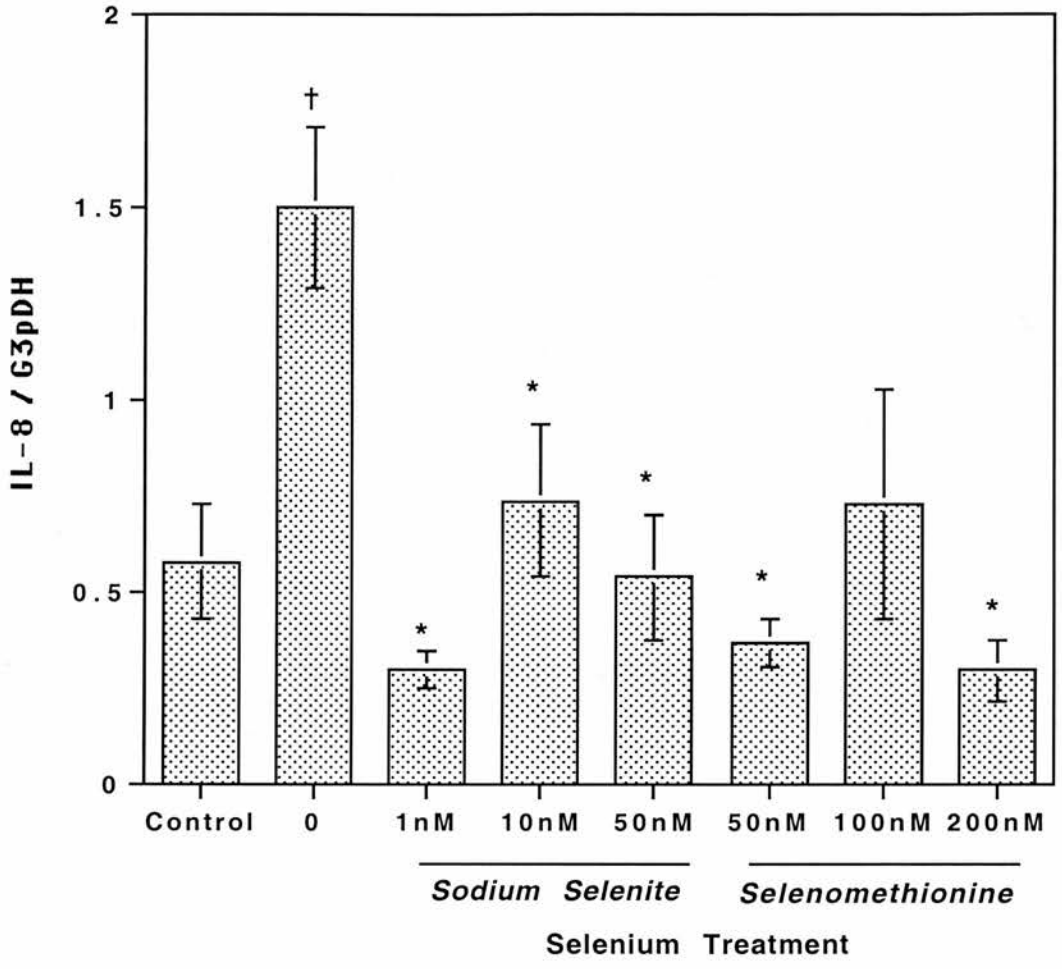
Figure 6.10b: Autoradiograph showing the effect of Se on the levels of IL-6 mRNA, following exposure to UVB.





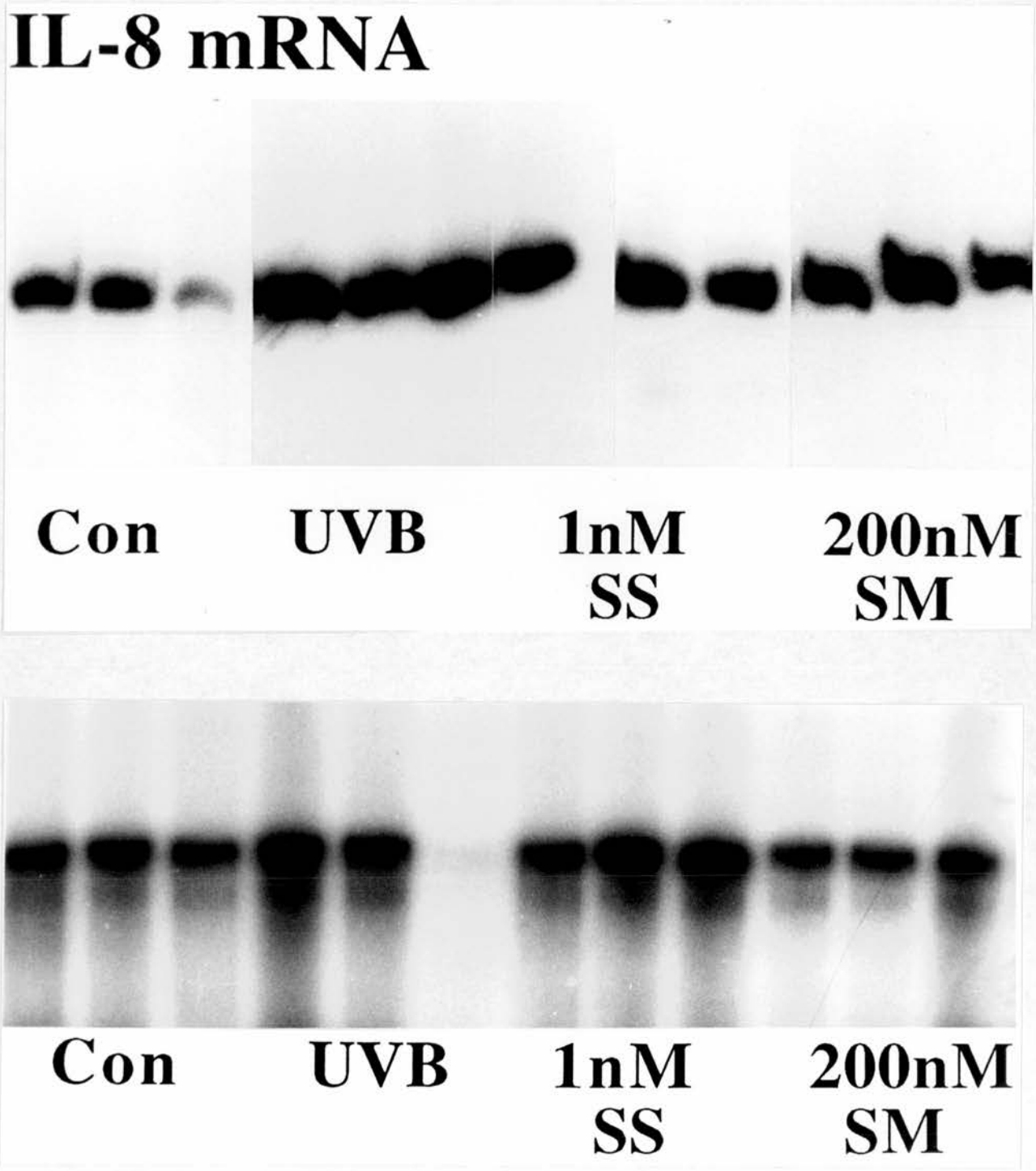
Autoradiographs of the SDS-PAGE gels for IL-6 mRNA (Top) and the housekeeping gene G3pDH (Bottom).

Figure 6.11a: Effect of Se on the levels of IL-8 mRNA, following exposure to UVB.



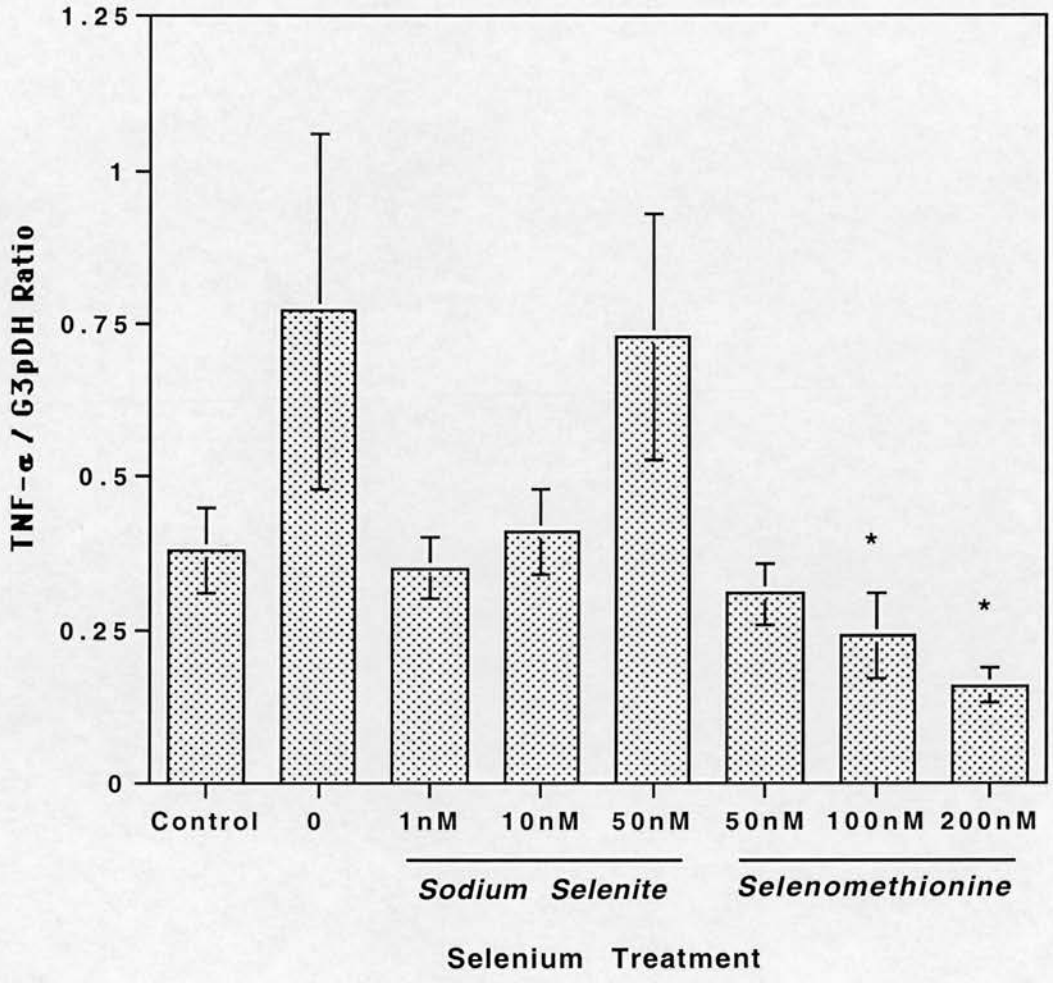
Primary keratinocytes were supplemented with either sodium selenite or selenomethionine for 24 hours prior to cells being exposed to UVB (200 J/m^2). Fresh media was returned to the cells and they were incubated for 6 hours before being harvested. The RNA was extracted and RT-PCR analysis was carried out on the samples using ^{32}P-labelled primers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. The cytokine levels were normalised using the housekeeping gene G3pDH. Control cells had no Se added and were mock irradiated. Results are expressed as the mean ratios ± S.E.M, n=3. Significant difference from the control cells, † = $P<0.05$, significant difference from the irradiated cells with no Se added * = $P<0.05$.

Figure 6.11b: Autoradiograph showing the effect of Se on the levels of IL-8 mRNA, following exposure to UVB.



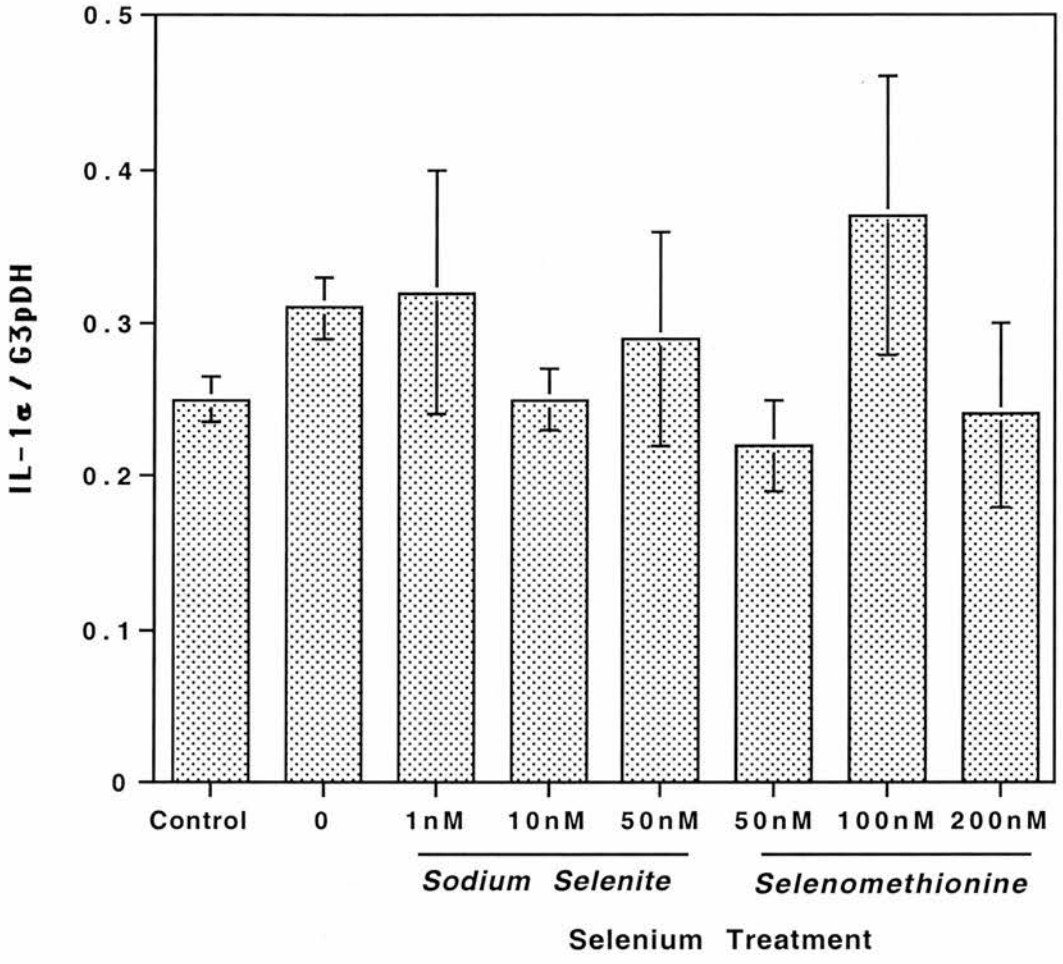
Autoradiographs of the SDS-PAGE gels for IL-8 mRNA (Top) and the housekeeping gene G3pDH (Bottom).

Figure 6.12: Effect of Se on the levels of TNF-α mRNA following exposure to UVB.



Primary keratinocytes were supplemented with either sodium selenite or selenomethionine for 24 hours prior to cells being exposed to UVB (200 J/m^2). Fresh media was returned to the cells and they were incubated for 6 hours before being harvested. The RNA was extracted and RT-PCR analysis was carried out on the samples using ^{32}P-labelled primers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. The cytokine levels were normalised using the housekeeping gene G3pDH. Control cells had no Se added and were mock irradiated. Results are expressed as the mean ratios ± S.E.M, n=3. Significant difference from the irradiated cells with no Se added * = $P<0.05$.

Figure 6.13: Effect of Se on the levels of IL-1α mRNA, following exposure to UVB.



Primary keratinocytes were supplemented with either sodium selenite or selenomethionine for 24 hours prior to cells being exposed to UVB (200 J/m^2). Fresh media was returned to the cells and they were incubated for 6 hours before being harvested. The RNA was extracted and RT-PCR analysis was carried out on the samples using ^{32}P-labelled primers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. The cytokine levels were normalised using the housekeeping gene G3pDH. Control cells had no Se added and were mock irradiated. Results are expressed as the mean ratios ± S.E.M, n=3.

6.2.1.5 Effect of Se on the UVB induction of mRNA for TNF-α and IL-10 by Pam 212 cells.

Pam 212 cells were grown in 6 well dishes and treated with various concentrations of Se for 24 hours and then exposed to UVB (200 J/m^2). After a further incubation of 6 hours, the cells were harvested and the RNA extracted and RT-PCR performed to quantify the mRNA for the cytokines of interest. The RT-PCRs were done using the cycle numbers described in section 6.2.1.1. The house keeping gene β-actin was used to normalise the levels of cDNA.

The mRNA for TNF-α was induced 6 hours following exposure to UVB. Sodium selenite diminished the induction of the mRNA at concentrations ranging from 10-200 nM and selenomethionine at concentrations ranging from 10-50 nM (Fig 6.14a and b).

The mRNA for IL-10 was not significantly induced 6 hours following exposure to UVB, this was due to the large differences between triplicate samples (Fig 6.15). Therefore it could not be determined if Se significantly decreased the mRNA for IL-10.

6.2.2 Cytokine Protein Levels.

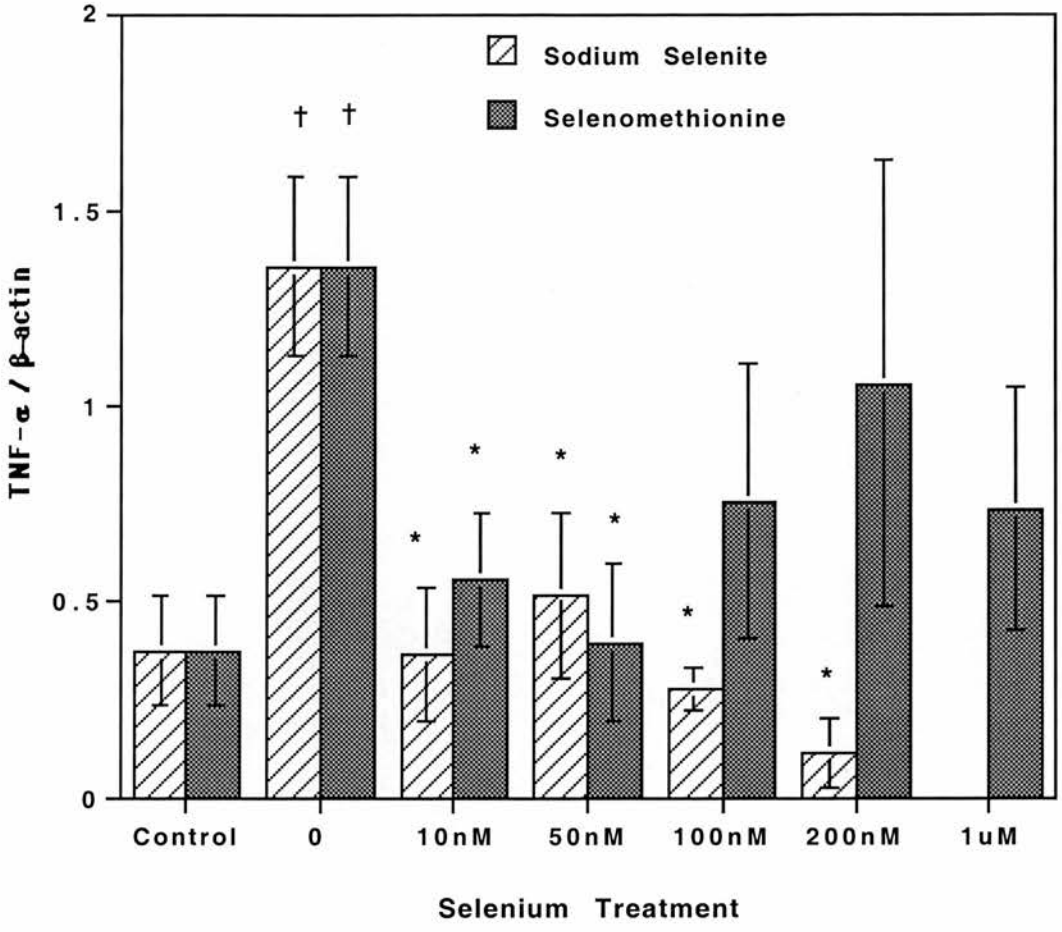
6.2.2.1 Effect of Se on basal protein levels of IL-6, IL-8 and TNF-α from primary keratinocytes.

The protein levels of IL-6, IL-8 and TNF-α were measured in the media from primary human keratinocytes using commercial ELISA kits. The cells were exposed to various concentrations of Se for 24 hours and then the media was collected and the protein levels in the media were measured by ELISA.

Sodium selenite did not decrease the basal levels of IL-6 protein; however selenomethionine did decrease the basal levels of IL-6 protein at 50 and 200 nM, however this reduction was not statistically significant (Fig 6.16). The

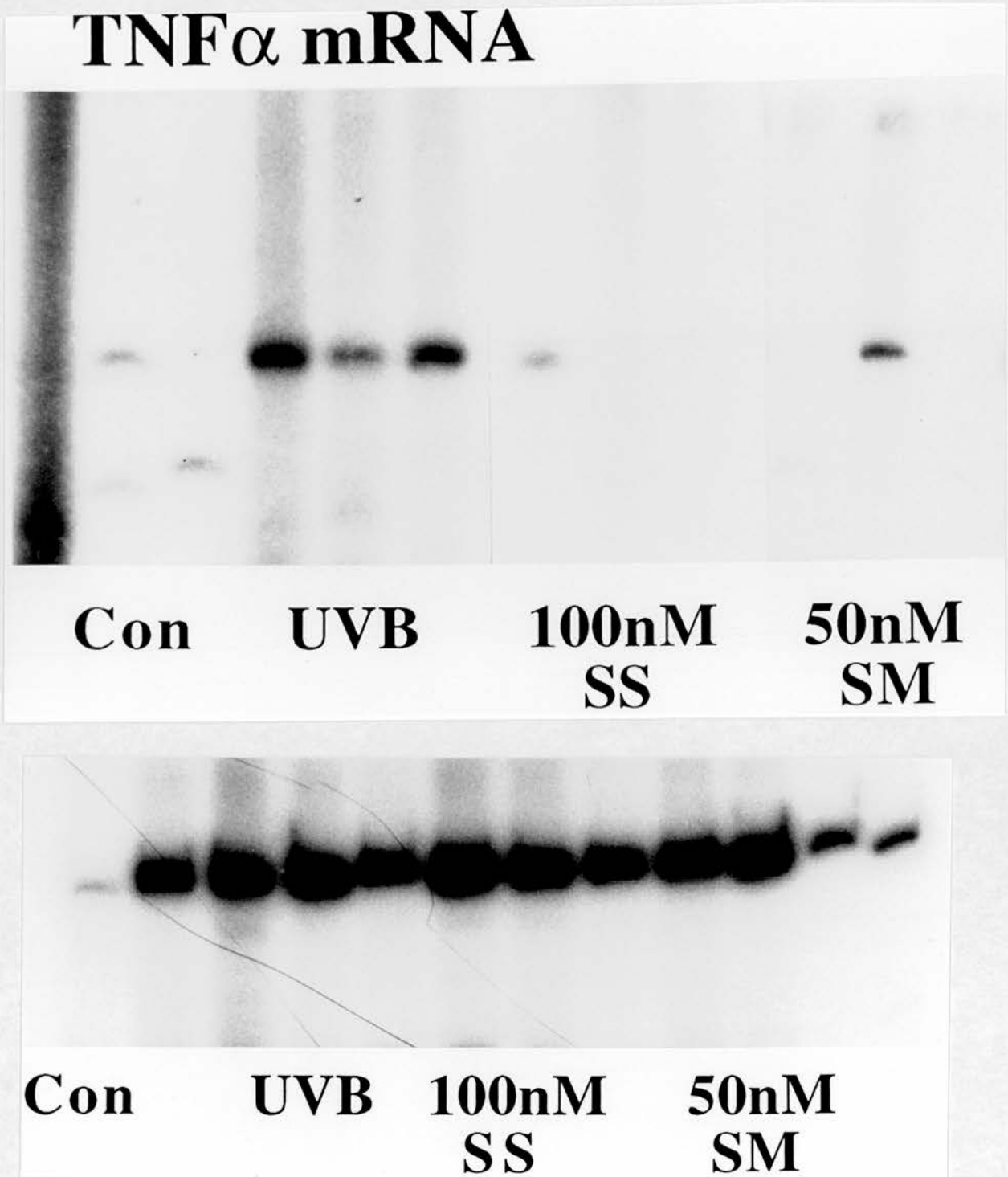
Continued on page 6-32

Figure 6.14a: Effect of Se on the levels of TNF-α mRNA in Pam 212 cells, following exposure to UVB.



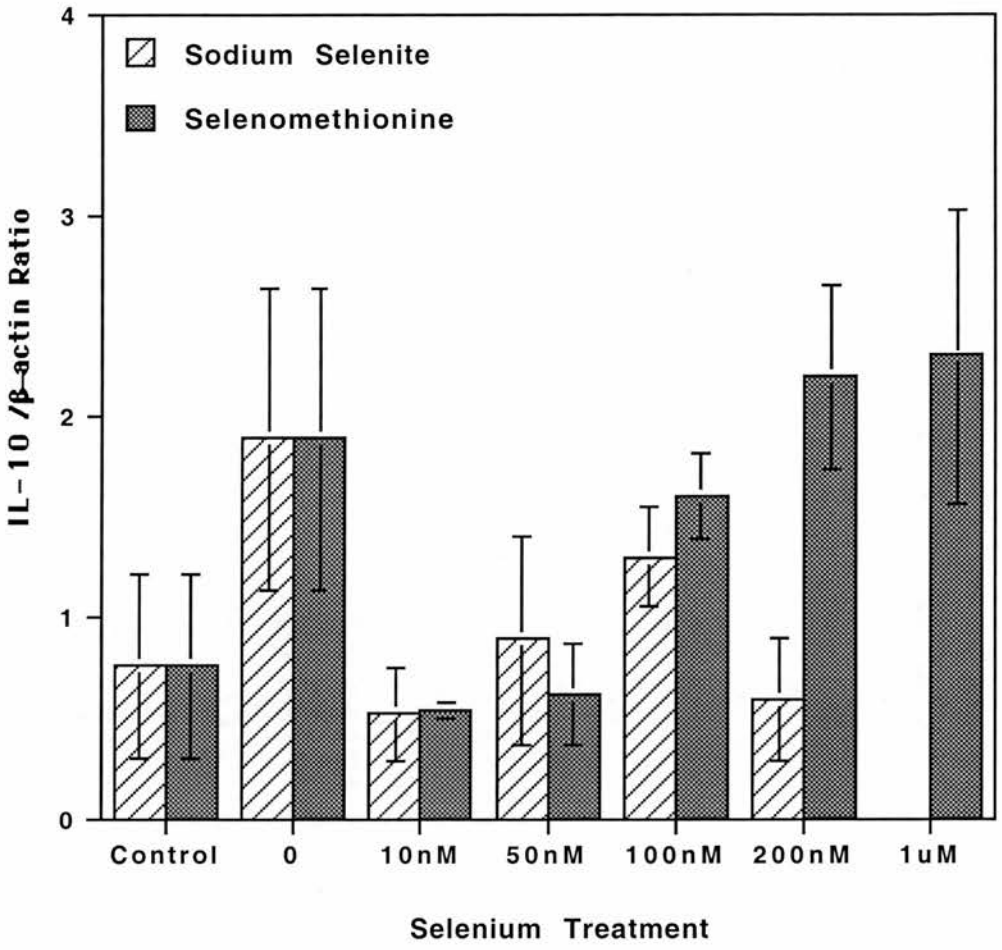
Pam 212 cells were supplemented with either sodium selenite or selenomethionine for 24 hours prior to cells being exposed to UVB (200 J/m^2). Fresh media was returned to the cells and they were incubated for 6 hours before being harvested. The RNA was extracted and RT-PCR analysis was carried out on the samples using ^{32}P-labelled primers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. The cytokine levels were normalised using the housekeeping gene β-actin. Control cells had no Se added and were mock irradiated. Results are expressed as the mean ratios ± S.E.M, n=3. Significant difference from the control cells, † = $P<0.05$, significant difference from the irradiated cells with no Se added * = $P<0.05$.

Figure 6.14b: Autoradiograph showing the effect of Se on the levels of TNF-α mRNA in Pam 212 cells, following exposure to UVB.



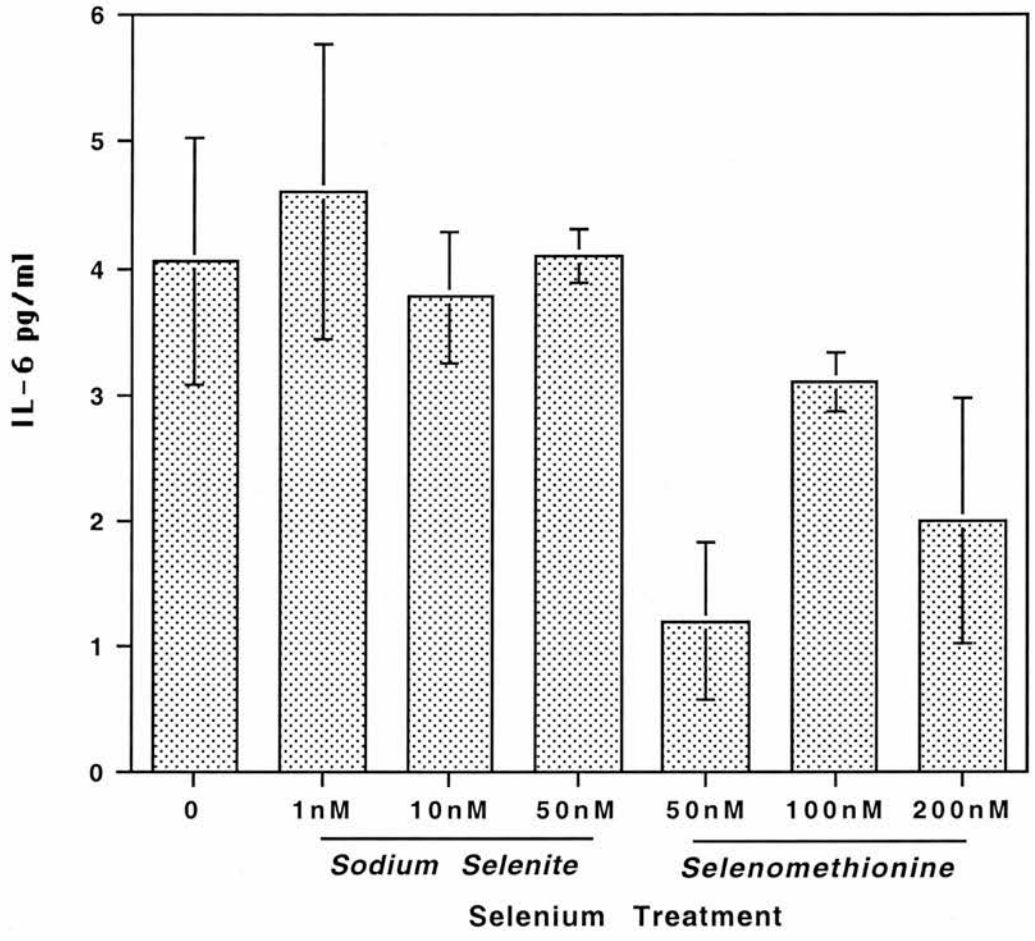
Autoradiographs of the SDS-PAGE gels for TNF-α mRNA (Top) and the housekeeping gene β-actin (Bottom).

Figure 6.15: Effect of Se on the levels of IL-10 mRNA in Pam 212 cells, following exposure to UVB.



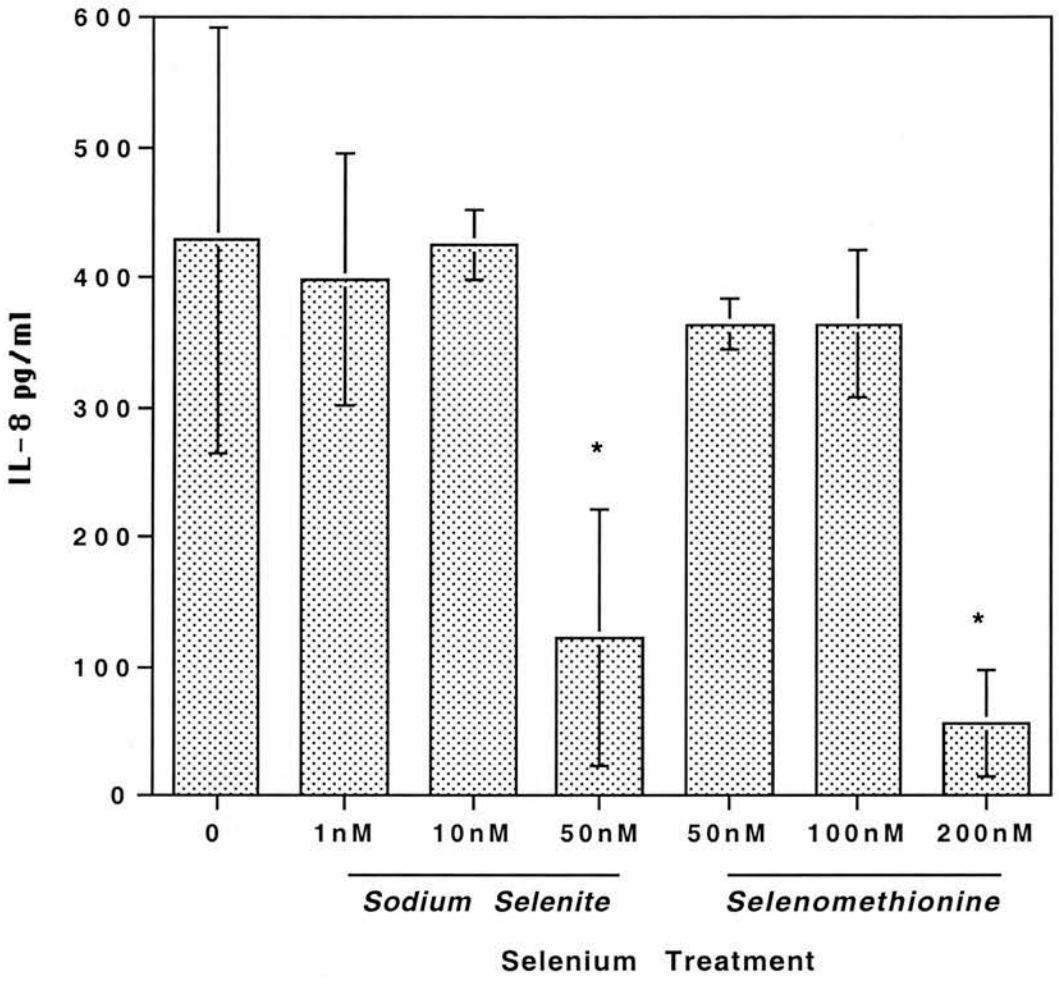
Pam 212 cells were supplemented with either sodium selenite or selenomethionine for 24 hours prior to cells being exposed to UVB (200 J/m^2). Fresh media was returned to the cells and they were incubated for 6 hours before being harvested. The RNA was extracted and RT-PCR analysis was carried out on the samples using ^{32}P-labelled primers. The PCR products were resolved on SDS-PAGE gels and quantitated using a phosphoimager. The cytokine levels were normalised using the housekeeping gene β-actin. Control cells had no Se added and were mock irradiated. Results are expressed as the mean ratios ± S.E.M, n=3.

Figure 6.16: Effect of Se on the basal levels of IL-6 protein.



Primary keratinocytes were supplemented with either sodium selenite or selenomethionine for 24 hours prior to the media being collected and the protein levels measured by ELISA. Control cells had no Se added. Results are expressed as the mean ratios ± S.E.M, n=3.

Figure 6.17: Effect of Se on the basal levels of IL-8 protein.



Primary keratinocytes were supplemented with either sodium selenite or selenomethionine for 24 hours prior to the media being collected and the protein levels measured by ELISA. Control cells had no Se added. Results are expressed as the mean ratios ± S.E.M, n=3. Significant difference from the control cells, * =$P<0.05$.

decrease in basal levels of the protein for IL-6 is in agreement with the effect of these concentrations of selenomethionine on the basal levels of IL-6 mRNA (Fig 6.9). Selenomethionine at 200 nM and sodium selenite at 50 nM significantly decreased the levels of IL-8 protein in the supernatants (Fig 6.17). This decrease contrasted with the effect of Se on the basal levels of mRNA, where all concentrations of Se significantly decreased the IL-8 expression (Fig 6.9).

The basal levels of TNF-α in the media were not affected by Se as there was no detectable TNF-α in unirradiated cells.

6.2.2.2 Effect of Se on protein levels for IL-6, IL-8 and TNF-α from primary keratinocyte cells exposed to UVB.

Exposure to UVB induced the release of IL-6 protein in primary keratinocytes at 24 hours following exposure. Pre-incubation of cells with various concentration of Se for 24 hours prior to exposure to UVB (200 J/m^2) did not affect the levels of protein released following exposure to UVB (Fig 6.18). This experiment was repeated three times in triplicate and Se did not affect the protein levels on any occasion.

There was significant induction of IL-8 protein at 24 hours following exposure to UVB, again Se did not alter the levels of protein induction. This experiment was also repeated three times in triplicate with similar results (Fig 6.19).

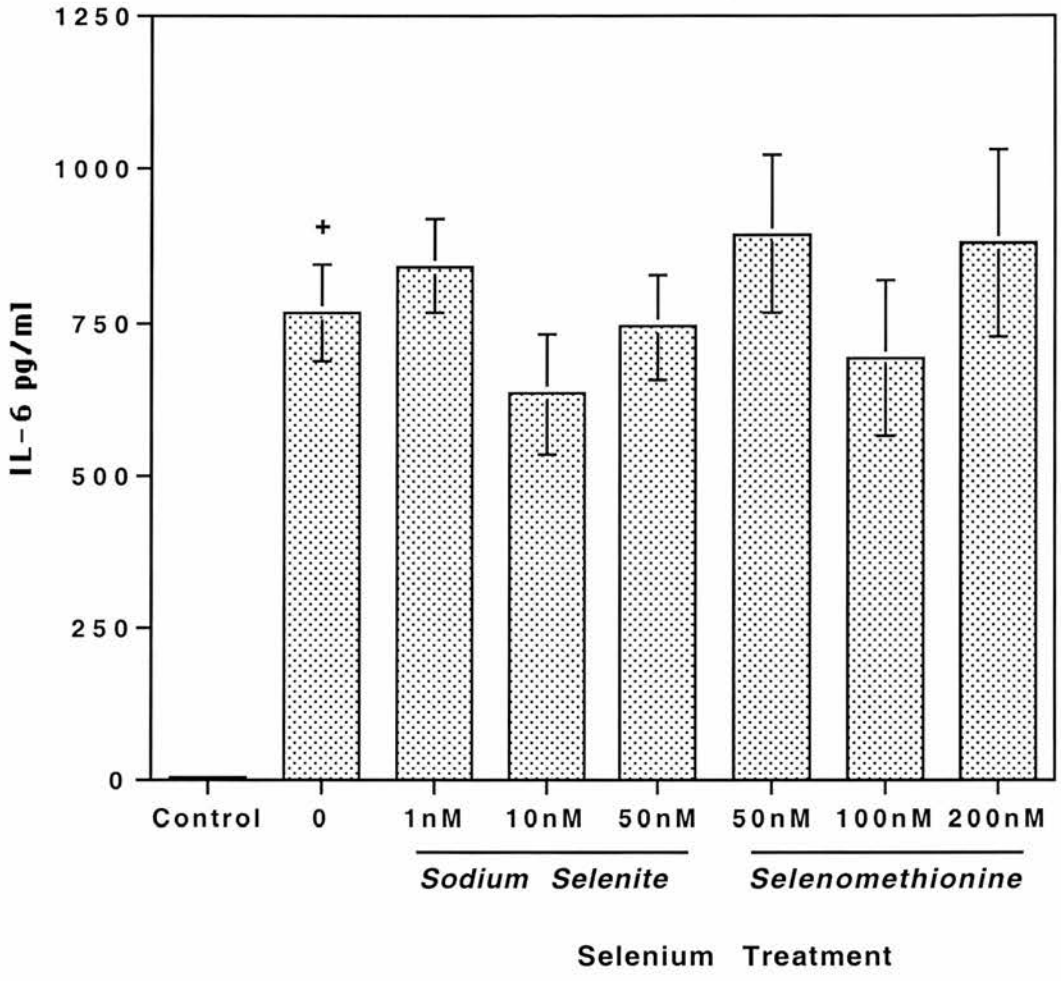
There was significant induction of TNF-α protein by exposure to UVB at 24 hours. There was a significant decrease in protein levels at 50 and 100 nM selenomethionine (Fig 6.20). However this experiment was only done once with triplicate samples. This was due to severe problems in stabilising the TNF-α protein during harvesting and storage, it was found that 0.1% foetal calf serum had to be added back to the media to stabilise the TNF-α protein in order that it would not deteriorate upon storage. Also the levels of TNF-α protein released were very low.

6.2.2.3 Effect of Se on protein levels for TNF-α and IL-10 from Pam 212 cells exposed to UVB.

The protein levels for IL-10 and TNF-α in the murine Pam 212 cells were investigated. However it was found that both proteins were undetectable using the currently available ELISA kits. However immunostaining was carried out on Pam 212 cells for IL-10. Pam 212 cells were cultured on glass coverslips and treated with various concentrations of Se for 24 hours before being exposed to 200 J/m^2 UVB. The cells were then incubated for a further 24 hours and then immunostained for IL-10. The full method is described in Chapter 2. The IL-10 staining shows as a brown colour in the cells. As in Chapter 4 with the p53 staining it was not possible to quantify the staining because unirradiated cells have low levels of IL- 10 present (Fig 6.21). Therefore the cells could only be graded for their IL-10 staining.

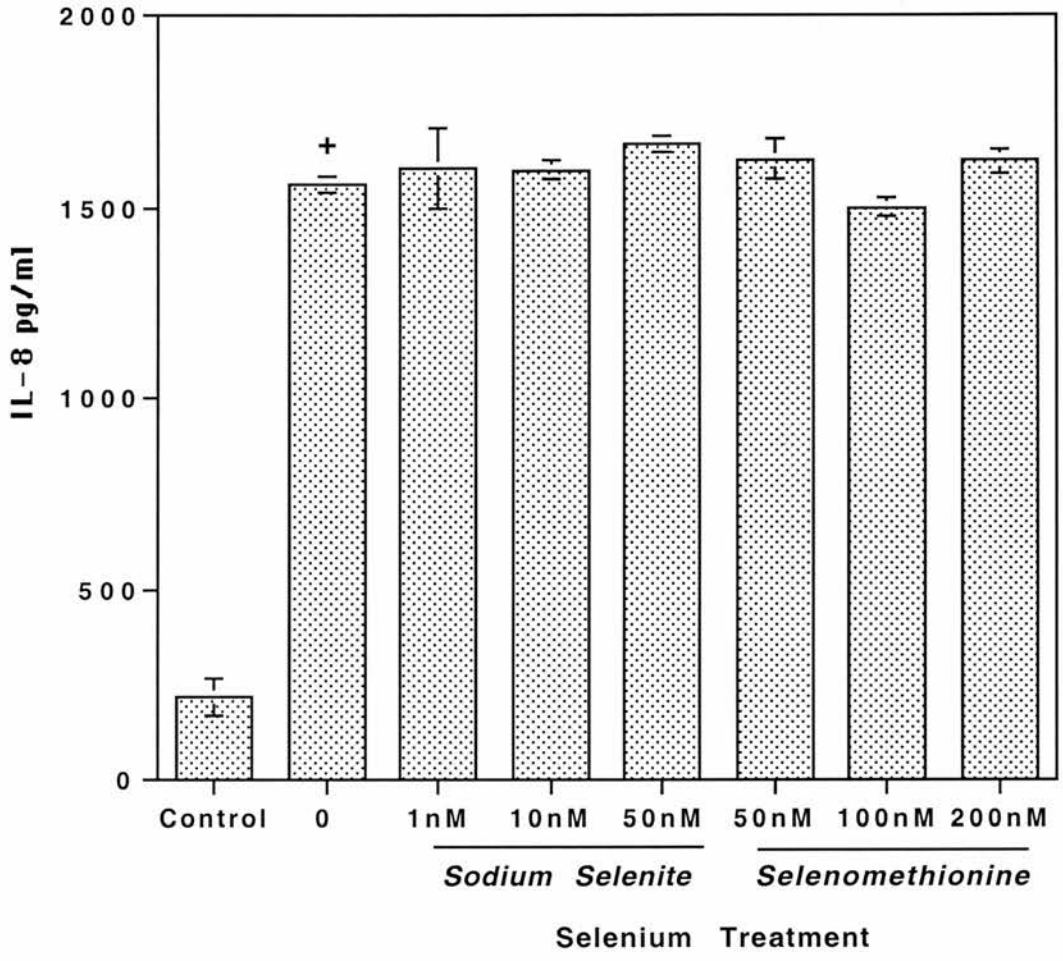
Exposure to 200 J/m^2 UVB increased the intensity of immunostaining for IL-10 protein in Pam 212 cells. Selenomethionine (50-200 nM) and sodium selenite (50 nM) were both found to decrease the intensity of immunostaining for IL-10 protein in Pam 212 cells (Fig 6.21 and Table 6.2). The largest decrease in immunostaining for IL-10 protein was found in the cells treated with selenomethionine.

Figure 6.18: Effect of Se on the levels of IL-6 protein, following exposure to UVB.



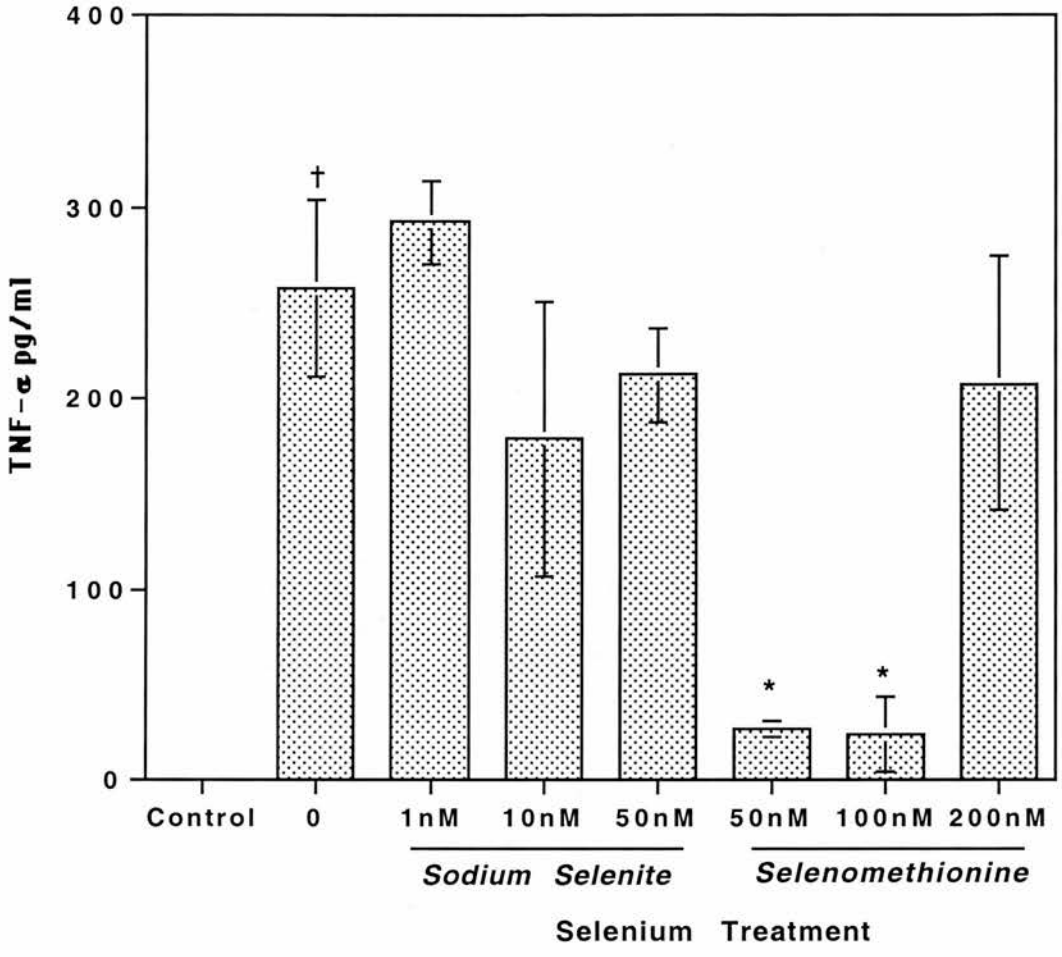
Primary keratinocytes were supplemented with either sodium selenite or selenomethionine for 24 hours prior to cells being exposed to UVB (200 J/m^2). Fresh media was returned to the cells and they were incubated for 24 hours prior to the media being collected. ELISA's were carried out on the samples. Control cells had no Se added and were mock irradiated. Results are expressed as the mean pg/ml ± S.E.M, n=3. Experiment was repeated in triplicate. Significant difference from the control cells, for the cells with no Se, but irradiated, † = $P<0.05$.

Figure 6.19: Effect of Se on the levels of IL-8 protein, following exposure to UVB.



Primary keratinocytes were supplemented with either sodium selenite or selenomethionine for 24 hours prior to cells being exposed to UVB (200 J/m^2). Fresh media was returned to the cells and they were incubated for 24 hours prior to the media being collected. ELISA's were carried out on the samples. Control cells had no Se added and were mock irradiated. Results are expressed as the mean pg/ml ± S.E.M, n=3. Experiment was repeated in triplicate. Significant difference from the control cells, for the cells with no Se but irradiated, † = $P<0.05$.

Figure 6.20: Effect of Se on the levels of TNF-α protein, following exposure to UVB.

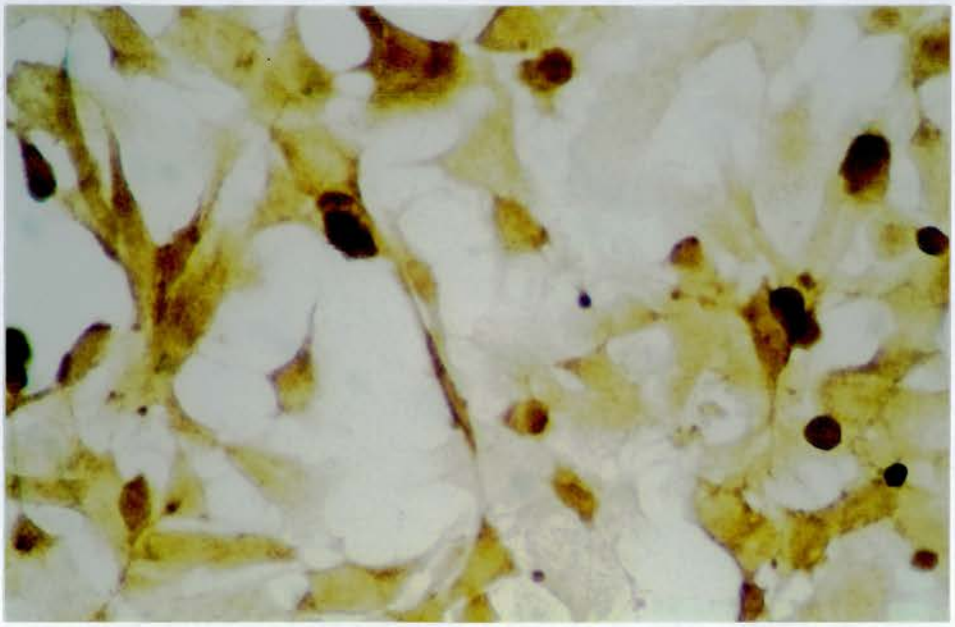


Primary keratinocytes were supplemented with either sodium selenite or selenomethionine for 24 hours prior to cells being exposed to UVB (200 J/m^2). Fresh media was returned to the cells and they were incubated for 24 hours prior to the media being collected. ELISA's were carried out on the samples. Control cells had no Se added and were mock irradiated. Results are expressed as the mean pg/ml ± S.E.M, n=3. The experiment was only carried out once. Significant difference from the control cells, for the cells with no Se but irradiated, † = $P<0.05$. Significant difference from the irradiated cells with no Se added * = $P<0.05$.

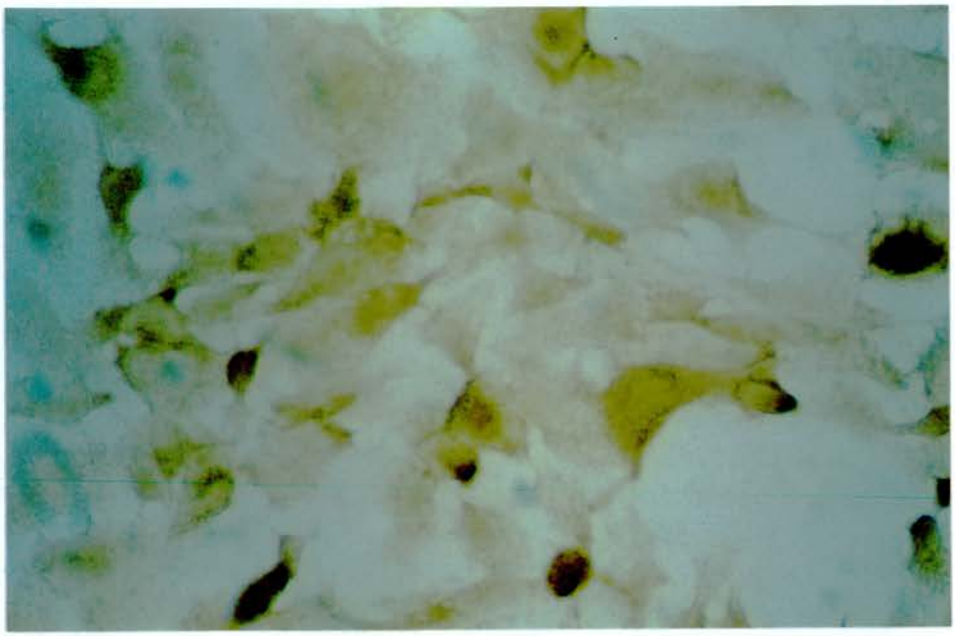
Figure 6.21: Effect of Se on immunostaining for IL-10 protein in Pam 212 cells.

a) Control cells (magnification x160)

b) Irradiated cells (200 J/m^2)



c) **Cells treated with selenomethionine (200 nM) and irradiated.**



d) **Cells treated with sodium selenite (50 nM) and irradiated.**

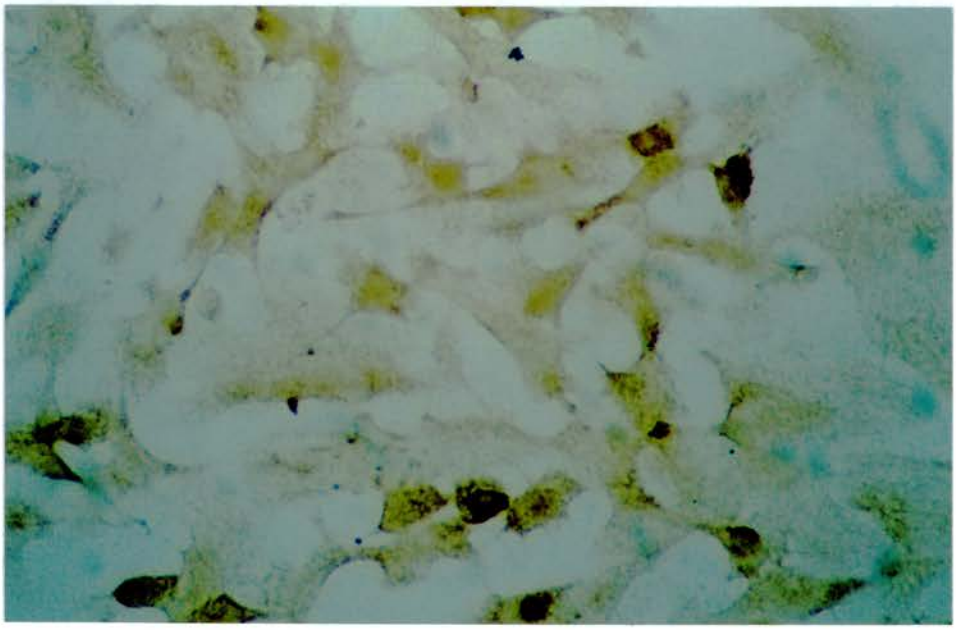


Table 6.2: Effect of Se on the UVB induction of IL-10 protein in Pam 212 cells, measured by immunostaining.

Treatment	Relative intensity of staining (individual experiments)		
Control	+	+	+
0	+++	+++	+++
1 nM sodium selenite	+++	+++	+++
10 nM sodium selenite	+++	+++	++
50 nM sodium selenite	++	+++	+
50 nM selenomethionine	++	++	++
100 nM selenomethionine	++	++	++
200 nM selenomethionine	+	++	+

The duplicate cultures were treated with sodium selenite or selenomethionine for 24 hours prior to the media being replaced and the cells exposed to 200 J/m^2 UVB. The original Se-containing media was then replaced and the cells incubated for a further 24 hours. The cells were immunostained for IL-10. Control cells were not treated with Se or irradiated. Results are the average intensity of staining, n=2, per experiment.

- = no staining
+ = faint staining
++ = stronger staining
+++ = very strong dark brown staining

6.3 Discussion.

Primary keratinocyte experiments.

6.3.1 Interleukin-6.

Production IL-6 mRNA was induced by exposure to UVB and peaked at 6 hours (Fig 6.5) whilst the protein production, peaked at 24 hours (Fig 6.18). This compares well with the literature, however others have reported that the peak in protein production normally occurs at 12 hours (Wlaschek *et al*, 1993; Kirnbauer *et al*, 1991). The basal levels of the mRNA and the protein were slightly decreased by selenomethionine at 50 and 200 nM (Fig 6.9 and 6.16). The decrease could be mediated by the effects of Se on the AP-1 and NFκB transcription factors by altering their binding to the IL-6 promoter region. These transcription factors have been shown to be regulated by the redox environment. NFκB can activate IL-6 mRNA production in keratinocytes and is itself activated by exposure to UVB light and can also be activated by free radicals (reviewed in Flohe *et al*, 1997). Overexpression of PHGPX in cells supplemented with 50 nM sodium selenite decreases the activation of NFκB by IL-1 and selenite treatment for 4 days also diminishes the level of NFκB DNA binding (Brigelius-Flohe *et al*, 1997). As Se has been demonstrated to decrease NFκB activation it is possible that this is the mechanism for the decrease in the levels of IL-6 mRNA and basal protein levels. The AP-1 is also activated by UV light (Derijard *et al*, 1994; Devary *et al*, 1991) and by H_2O_2. Selenite and selenodigluthione added to nuclear extracts effectively inhibit AP-1 DNA binding (Spyrou *et al*, 1995). Therefore this represents another possible mechanism for the decrease in basal levels of IL-6. However it does not explain why selenomethionine but not selenite decreases the basal level of IL-6.

When keratinocytes were supplemented with Se and then exposed to UVB conflicting results arose. The level of mRNA induced by UVB was decreased at all the concentrations of selenomethionine used and also at 1 nM sodium selenite (Fig 6.10a and b). However the levels of protein did not change (Fig 6.18). There are several possible explanations to account for this difference, the increase in the abundance of mRNA for IL-6 following exposure to UVB appears to be unimportant. The increase in IL-6 protein appears to be due to

an increase in mRNA stability and not increased transcription (de Vos *et al*, 1990). It may be the case that the presence within the IL-6 promoter region of NFκB and AP-1 binding sites, is not important for the response to UVB. Furthermore the Se-mediated decrease in transcription factor activation may not overcome other actions which lead to the stabilisation of the mRNA for IL-6 . The mRNA for IL-6 has a conserved AU-rich region, found in the 3' untranslated region, which normally causes destabilisation of the mRNA (Beelman and Parker, 1995; Caput *et al*, 1986; Malter, 1989). During inflammation the mRNA for IL-6 is stabilised through binding of cytosolic proteins, which specifically recognise the AUUA rich structural elements and prevent degradation of the mRNA (Malter, 1989). Moreover, it was reported that UV irradiation enhances the cross-linking between the AU rich sequence elements and their protective binding proteins (Henics *et al*, 1995). It maybe that Se decreases the production of the mRNA for IL-6. However it is clear that the decrease in mRNA does not lead to a decrease in the level of IL-6 protein induced following exposure to UVB. Therefore a threshold of stabilised mRNA may be reached that allows the production of increased levels of protein. Another possibility is that Se may stabilise the cytokine proteins, perhaps by inhibiting the action of proteases.

6.3.2 Interleukin-8.

The levels of mRNA and protein for IL-8 were increased by exposure to UVB 6 (Fig 6.6) and 24 hours respectively (Fig 6.19). This increase in both mRNA and protein following exposure to UVB is in agreement with other reports in the literature (Kondo *et al*, 1993). The basal levels of IL-8 protein were decreased by addition of 200 nM selenomethionine and 50 nM sodium selenite (Fig 6.17), and the basal level of mRNA was decreased at most concentrations of Se (Fig 6.9). The decrease in mRNA and protein for IL-8 may be caused by the influence Se has on transcription factors activation and on ROS-mediated signal transduction. The effect of Se on two of the transcription factors has been described in section 6.3.1. Protein levels for IL-8 were not decreased to the same degree as mRNA levels when Se was added, this may demonstrate the presence of a surplus of IL-8 mRNA in the cells.

Again when the cells were supplemented with Se and then exposed to UVB conflicting results arose. The levels of mRNA induced by UVB were diminished at 50 nM and 200 nM selenomethionine and at all concentrations of sodium selenite (Fig 6.11a and b), however the levels of protein did not decrease (Fig 6.19).

Kondo et al have suggested that the induction of IL-8 mRNA is due to AP-1 activation mainly, because the mRNA is induced within 1 hour and the proteins c-fos and c-jun which make up the transcription factor AP-1 are also turned on rapidly by UVB (Shah *et al*, 1993; Kondo *et al*, 1993). Most of the induction of IL-8 following UVB irradiation is due to *de novo* synthesis of the protein (Kondo *et al*, 1993). The induction of mRNA via AP-1 activation may explain why the levels of mRNA are induced by UVB and decreased by Se. However it does not account for the lack of inhibition in protein levels with Se. There may be sufficient mRNA, encoding for IL-8 even after it is decreased by Se, to allow efficient production of the protein.

6.3.3 Tumour necrosis factor-α.

The level of mRNA for TNF-α was increased by exposure to UVB at 6 hours (Fig 6.7), but the induction was not significant, however the protein was induced significantly at 24 hours (Fig 6.20). The time points for peak induction agree with those published in the literature (Kock *et al*, 1990). The basal level of TNF-α mRNA was not decreased by any of the concentrations of Se employed (Fig 5.9). Whilst TNF-α does have AP-1 and NFκB binding sites within it's promoter region (Spriggs *et al*, 1992); if these sites are mutated induction of the gene can still occur (Bazzoni *et al*, 1994). This would suggest that these transcription factor sites are not absolutely necessary for induction of the message. Therefore if Se does indeed decrease the activation of these two transcription factors, this would not necessarily decrease the level of TNF-α mRNA.

When the primary human keratinocytes were supplemented with Se and then exposed to UVB, the levels of mRNA induced by UVB were decreased at 100 and 200 nM selenomethionine. Sodium selenite may have decreased the mRNA significantly, however there was no significant induction of TNF-α mRNA (Fig 6.12). The levels of TNF-α protein were also decreased

significantly at 50 and 100 nM selenomethionine (Fig 6.20). There are several possible reasons for this, it has been demonstrated that antioxidants and can reduce the amount of TNF-α production following exposure to UVB (Corsini *et al*, 1995). This suggests that ROS are a major inducer of TNF-α protein following UVB exposure. If Se lead to an increase in the antioxidant selenoproteins GPX and TR this would lead to a decrease in ROS-mediated induction of the mRNA for TNF-α, which in this case appears to lead to a decrease in the abundance of TNF-α protein. However the TNF-α ELISA experiment was only carried out once due to problems with the stability of the protein. The absence of any decrease in TNF-α protein levels after sodium selenite pretreatment requires further investigation.

The decrease in TNF-α protein is of interest in the context of the skin where it acts as a pro-inflammatory cytokine which can stimulate IL-1, IL-8 and IL-6 release by keratinocytes (Kutsch *et al*, 1993). Therefore if Se reduces the induction of TNF-α protein within the skin this could lead to a concomitant abrogation of the inflammatory response. Also TNF-α is implicated in LC migration from the skin following exposure to UVB. The migration of LCs from the epidermis leads to local immune suppression, which may allow altered self antigens and pathogens to evade the immune system. Therefore any reduction in TNF-α release caused by Se would prevent LCs from receiving their migratory signal. Indeed, as discussed in Chapter 8 there is a greater decrease in the numbers of LCs in Se-deficient mice then in Se-replete mice.

6.3.4 Interleukin-1α.

The IL-1α mRNA was not significantly induced by UVB (Fig 6.8) and Se had no effect on its basal levels before or after exposure to UVB (Fig 6.9). It was decided not to pursue an investigation of the protein levels of IL-1α, which is released 12 hours following exposure to UVB. The release of IL-1α is not due to *de novo* protein synthesis, *de novo* transcription or to mRNA stabilisation, as UVB decreases the half-life of the mRNA (Kondo *et al*, 1994). The increase in IL-1α protein is accounted for by the release of performed IL-1α. Therefore it was not thought that Se would affect these protein levels.

6.4 Pam 212 cell line experiments.

6.4.1 Tumour necrosis factor-α.

In Pam 212 cells the induction of mRNA for TNF-α was significant 6 hours post exposure to UVB. Selenomethionine at concentrations of 10-50 nM and sodium selenite at concentration of 10-200 nM significantly decreased this induction following exposure to UVB (Fig 6.14a and b). As in the case of human TNF-α this is probably due to a decrease in transcription factor activation and therefore a decrease in mRNA transcription. As described in section 6.3.3.

Unfortunately TNF-α protein could not be detected with currently commercially available ELISA kits. The role of Se in modulating the production of murine TNF-α protein could not be investigated.

6.4.2 Interleukin-10.

The increase in IL-10 mRNA following exposure to UVB was found not to be significant, therefore this prevented a study of the full effects of Se on the levels of IL-10 mRNA (Fig 6.15). However sodium selenite (1-100 nM) and selenomethionine at concentrations between 10-100 nM did appear to decrease the levels of mRNA although this decrease did not reach significance.

The IL-10 protein was undetectable by the currently commercially available ELISA kits. However there was a monoclonal antibody available for murine IL-10 and immunostaining for the protein was carried out. It was found that selenomethionine at concentrations between 50-200 nM, and sodium selenite at 50 nM decreased the level of IL-10 protein (Table 6.2). It was also attempted to carryout western blotting unfortunately the antibody did not work when using this method.

The decrease in IL-10 protein levels following exposure to UVB, could lead to a reduction in the level of systemic immune suppression induced by UVB and a stronger cytotoxic cellular immune response (reviewed in Kondo and Sauder, 1995). Also IL-10 has been implicated in inhibiting antigen

presentation within the skin and so a decrease in IL-10 would lead to improved antigen presentation by LCs in the skin (Beissert *et al*, 1995; Niizeki and Streilein, 1997; Barr *et al*, 1999).

6.5 Summary.

There is currently little evidence that Se can affect the levels of cytokines in the skin. Selenium supplementation can reduce the release of inflammatory cytokines from the skin (Celerier *et al*, 1995). The decrease in the protein levels maybe due to a decrease in the activity of transcription factors and cellular signalling pathways, such as activation of phospholipases by Se (Brigelius-Flohe *et al*, 1997; Spyrou *et al*, 1995). Previous studies have demonstrated that a diet deficient in Se is associated with an increased inflammatory response (reviewed in McKenzie *et al*, 1998). This increase in response may be due to the lower level of antioxidant selenoproteins present leading to an increased level of free-radicals, which could activate transcription factors and signalling pathways (Devary *et al*, 1992; Simon *et al*, 1994). Unfortunately the work presented in this chapter does not really provide a full explanation for the decrease in inflammation elicited by Se. While Se does decrease the levels of mRNA for; IL-6, IL-8, TNF-α in human cells and IL-10, TNF-α in murine cells. Addition of Se only decreased the levels of UVB-induced TNF-α protein from primary keratinocytes and IL-10 protein from murine cells. The decrease in TNF-α protein is not enough to explain how the inflammatory response to UVB is diminished by Se. Some alternative possible mechanisms include a decrease by Se in the production of inflammatory free-radicals and prostaglandins. Selenium may also lower the levels of iNOS protein and therefore, nitric oxide release. Keratinocytes demonstrate enhanced prostaglandin secretion following UVB exposure, in particular, PGE_2 which is also believed to contribute to UVB-induced inflammation. Selenium supplementation has been reported to decrease the induction of prostaglandins (reviewed in McKenzie *et al*, 1998).

The decrease in IL-10 protein release found in the murine cells is of interest as following UVB exposure, systemic and local immune suppression can be induced. The immune suppression allows tumour antigens to evade the immune response, thereby allowing progression of skin tumours.

6.6 Further Work.

There is a great deal of further work which needs to be undertaken in this area to gain an understanding of how Se affects the immune system. The TNF-α protein and mRNA experiments need to be repeated as the protein experiments in the human cells were performed once. The effect of Se on the activation of transcription factors following exposure to UVB requires further study. The mechanisms by which the mRNA of cytokines increase following exposure to UVB needs to be investigated. The effect of Se on mRNA stabilisation and protein stability. Finally the effect of Se on other inflammatory mediators such as prostaglandins, nitric oxide or free radicals warrants further examination.

Chapter 7

Effect of Selenium on Epidermal Langerhans' Cell Numbers in Mice.

7.1 Introduction.

7.1.1 Immune suppression.

The phenomenon of UV-induced modulation of tumour immunity was first noticed by Margaret Kripke and co-workers in 1977, who reported that UV-induced skin tumours, transplanted to normal syngenic mice, were rejected by these immunocompetent hosts. However, the tumours grew progressively if the recipient had been irradiated, with UV radiation at subcarcinogenic doses, prior to transplantation (Fisher and Kripke, 1977; Kripke, 1976; Kripke, 1984). Therefore, UVB appears to have a role in both tumour initiation by causing DNA damage and as a tumour promoter by inhibiting tumour surveillance and hence, can be described as a complete carcinogen.

7.1.2 Langerhans' cells.

Langerhans' cells (LCs) are CDla+, MHC class II+ dendritic cells, derived from the bone marrow. Langerhans' cells form a semi-continuous network between epidermal keratinocytes and function as the main antigen presenting cells (APCs) in the skin. Langerhans' cells are described in more detail in Chapter 1.

7.1.3 Effect of UVB on Langerhans' cells.

Invading pathogens, contact sensitisers or UVB exposure can all induce LCs to migrate from the epidermis (Toews *et al*, 1980, reviewed in Meunier, 1999). Many cytokines are upregulated by exposure to UVB including IL-1α, IL-6, IL-8, IL-10, TNF-α and TGF-β.

TNF-α has been proposed to initiate the movement of LCs from the skin to the draining lymph nodes (Cumberbatch and Kimber, 1995). The encoding mRNA and the TNF-α protein are upregulated in the epidermis, after exposure to UVB. Furthermore, intradermal injection of TNF-α causes an accumulation of dendritic cells in draining lymph nodes (Cumberbatch and Kimber, 1992) and a decrease in LCs numbers in the epidermis (Cumberbatch *et al*, 1994). These effects implicate TNF-α as an important mediator in LC cell migration. Furthermore, pre-treatment of mice with neutralising antibodies for TNF-α before UV exposure, prevents the normal UV-induced accumulation of dendritic cells in the draining lymph nodes.

Interleukin-1β has also been implicated in the migration of LCs to the draining lymph nodes (Cumberbatch *et al*, 1997). It is thought that IL-1β is mainly LC-derived, and it can upregulate epidermal TNF-α (Enk *et al*, 1993). As the LCs move to the draining lymph nodes they undergo many changes. One important change is that LCs express less E-cadherin which is thought to help LCs bind to keratinocytes (Tang *et al*, 1993). The down regulation of E-cadherin may enable LCs to detach from keratinocytes.

Both sub-erythemal and erythemal doses of UVB cause a decrease in the number of LCs in human skin, this decrease can be between 20-70% respectively (Cooper *et al*, 1992). In addition, morphological changes occur to the LCs including; the loss of dendrites and the alteration of cell surface markers (Aberer *et al*, 1981). UVB can reduce the expression of ICAM-1 on LCs (Tang and Udey, 1991). UVB exposure also affects LC function. For example it inhibits their ability to stimulate primary alloresponses and their proliferation in response to mitogens and antigens (Rattis *et al*, 1995a and b).

Free radicals appear to play a role in UVB-induced damage to LCs. When SOD is injected intradermally into guinea pigs just prior to their exposure to UVB, the LC population in the skin is significantly protected (Horio and Okamoto. 1987). Glutathione has also been reported to preserve the antigen presenting ability of LCs (Iwai *et al*, 1999). The protective effect of SOD and glutathione suggests that oxygen radicals may damage LCs in the skin following exposure to UVB. Recently it has been established that LCs can undergo apoptosis following exposure to UVB (Rattis *et al*, 1998). It has also been demonstrated that the DNA of LCs can be damaged following

exposure to UVB. The damage caused is typical of the effects of exposure to UVB, and includes the formation of thymidine dimers (Sontag *et al*, 1995).

7.1.4 Effect of Se on the immune system.

Selenium has been demonstrated to play an important role in the immune system (reviewed in McKenzie *et al*, 1998). Deficiency of dietary Se is reported to reduce antibody titres in mice (Spallholz *et al*, 1973, 1975, 1990). A more recent study found that Se-deficient rats have lower antibody titres and a decreased delayed type hypersensitivity reaction (Kukreja and Khan, 1998). Selenium can also upregulate the expression of the T cell high affinity IL-2 receptor. This increases T cell responsiveness to IL-2 and therefore T cell proliferation and function (Roy *et al*, 1995). Cytotoxic T cell function and natural killer cell activity are also boosted by Se supplementation (Meeker *et al*, 1985; Koller *et al*, 1985; Petrie *et al*, 1989a and b; Kiremidjian-Schumacher *et al*, 1992 and 1996). Erythema induced by exposure to UVB and inflammatory cytokine expression in inflamed skin in organ cultures, can be diminished by Se supplementation (Celerier *et al*, 1995). The role of Se in the immune system is discussed in more detail in Chapter 1.

Whilst Se does affect the immune system, as yet little is known about how it modulates cytokine expression, inflammation, UV-induced immune suppression and antigen presentation.

7.1.5 Measurement of Se Status *in vivo*.

The most common method for assessing Se status is to measure the activity of cGPX in erythrocytes or plasma, since GPX activity depends on Se status (Rotruck *et al*, 1973). The activity of cGPX decreases in response to Se deficiency at different rates in different tissues i.e. the endocrine tissues and brain retain their levels of Se during Se deficiency. This produces a hierarchy of Se utilisation, such that tissues which retain their Se levels are thought to utilise it for more essential functions (reviewed in Burk and Hill, 1993; Allan *et al*, 1999). For example, in the liver cGPX activity can fall to <1% in Se-deficient animals compared to levels in Se-replete animals, with no short term adverse health effects. The effect of Se deficiency on lymph

node and skin levels of cGPX are not known, therefore cGPX measurements were examined.

The aims of this study were to :-

- Ensure that the different Se levels in the diets used in this study did not affect the growth rates of the mice.

- Investigate the effect of exposure to UVB on the numbers of LCs present in the skin.

- Demonstrate the effect of Se on the number of LCs present in the skin prior to and following exposure to UVB.

- Study the effect of the different Se dietary intakes on the GPX activity in the draining lymph nodes, skin and livers of the mice.

- Demonstrate the effect of UVB on the GPX activity in the skin, liver and lymph nodes of the mice.

7.2 Methods and Results.

7.2.1 Effect of diets containing different levels of Se on the growth of mice.

The mice (C3H/HeN) were weaned at 3 weeks, and were maintained on three different diets, each containing different levels of Se (0ppm, 0.1ppm and 2ppm), for 6 weeks prior to the experiments being performed. The source of the mice and the dietary information is described in full in Chapter 2, section 2.7.1 and 2.7.2. Preliminary experiments were also carried out to ensure that the mice increased in weight, at a similar rate to mice fed on the normal animal house diet (Fig 7.1a). During the six weeks the mice were weighed every week. No differences in weight gain between the three Se diets were found (Fig 7.1b).

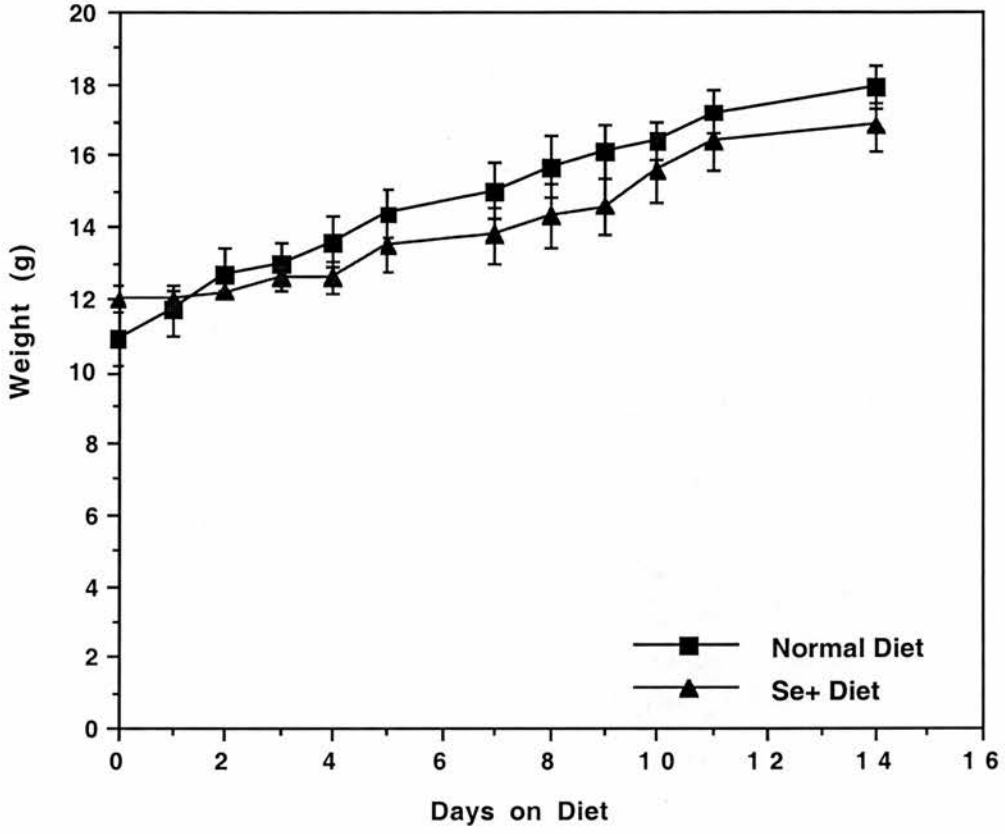
7.2.2 Effect of dietary Se levels on the numbers of LCs present in the epidermis.

After feeding the mice on one of the three Se diets for six weeks, 4 mice from each dietary group were irradiated with UVB (1440 J/m^2). The mice were exposed to UVB (TL-20W/12 lamps) in a perspex box, with a maximum of four mice per box to avoid shielding by litter mates. Twenty-four hours later, one ear from each mouse was harvested and stained for LCs, by assessing adenosine triphosphatase (ATPase staining) (see Chapter 2, section 2.7.3 for full methodology). Figure 7.2 illustrates ATPase-stained LCs in the epidermis. The cells can be distinguished by their dark brown/black staining and their dendritic appearance.

In Figure 7.3 it can be seen that the level of dietary Se, significantly modulated the number of LCs found in unirradiated skin. The mice maintained on the 0.1ppm Se diet had significantly increased numbers of LCs present, compared to the Se-deficient mice. The higher levels of LCs were found in three individual experiments, two of which are illustrated in Fig 7.3a and 7.3b. The experiments using the diets with 2ppm Se were performed twice. In the first experiment the mice fed with 2ppm diets had

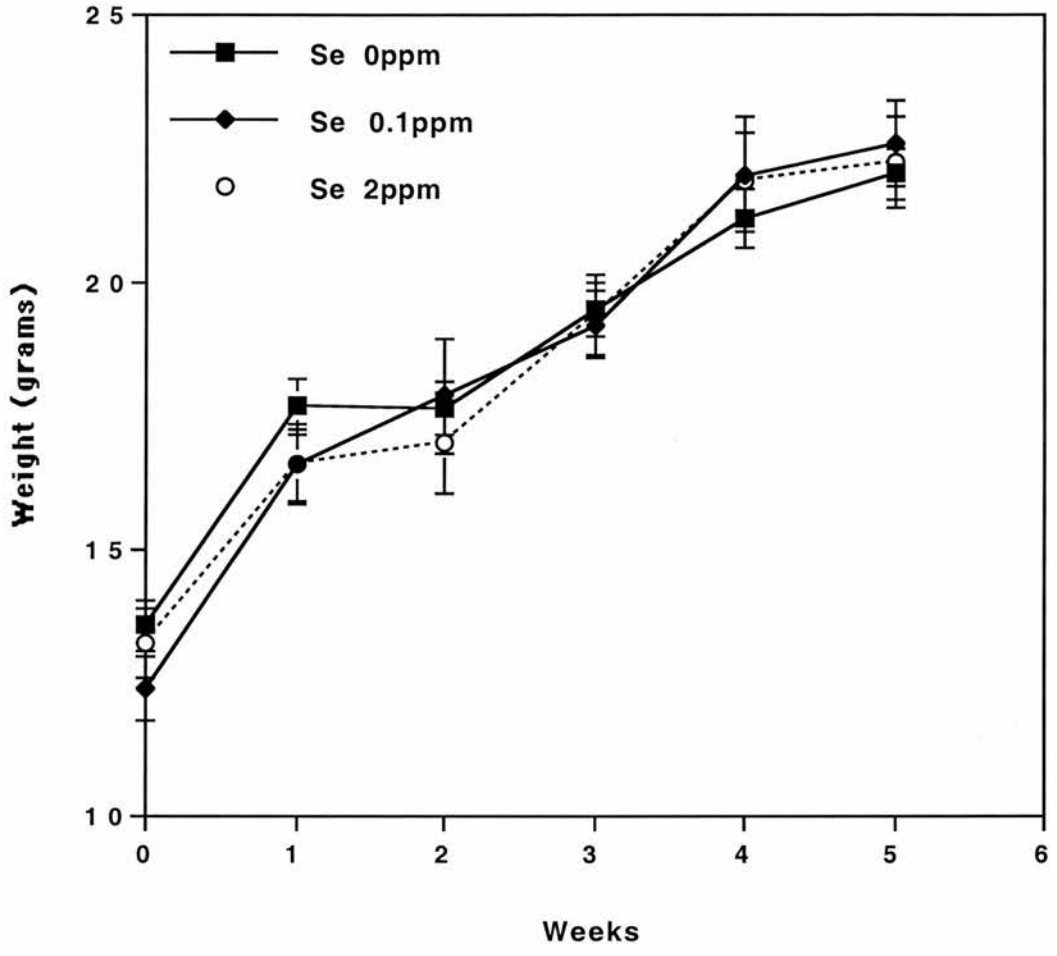
Continued on page 7-11

Figure 7.1a: Effect of Se diet on weight gain in C3H/HeN mice.



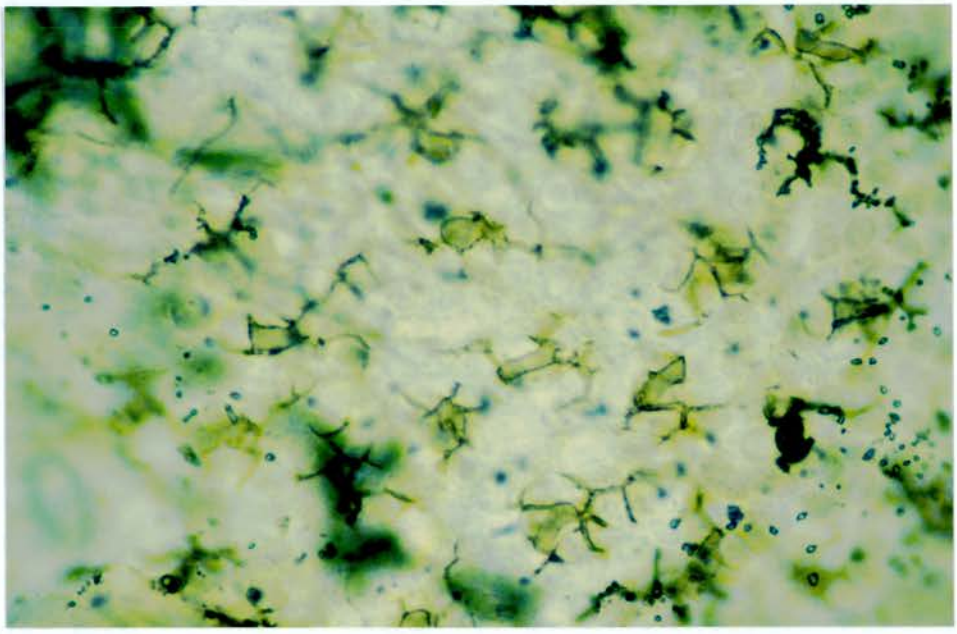
C3H/HeN mice were weaned at 3 weeks, and were fed on a special diet containing 0.1ppm Se or on the normal mouse house facility diet. Fresh food was given every 2 days. Mice were maintained for 2 weeks and were weighed daily. Results are the mean weights ± S.E.M (n=6).

Figure 7.1b: Effect of level of dietary Se on weight gain in C3H/HeN mice.



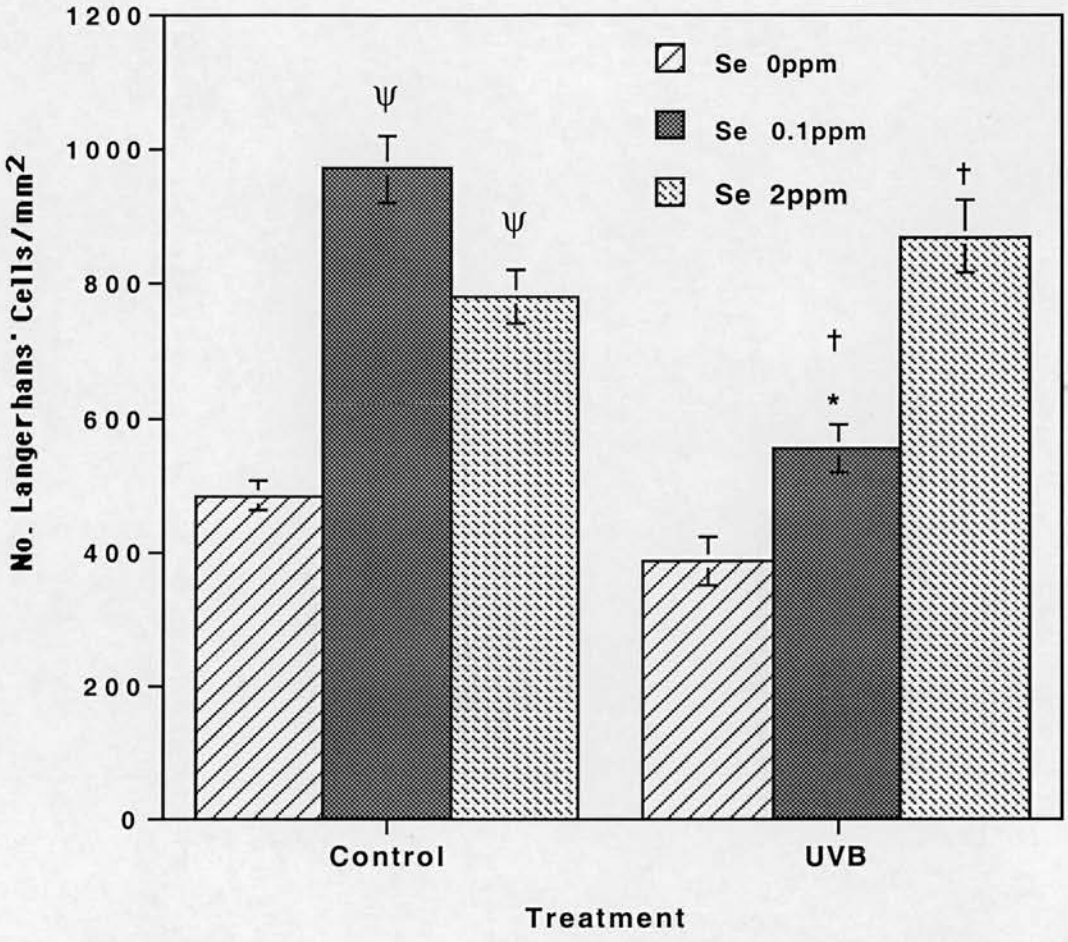
C3H/HeN mice were weaned at 4 weeks, and were fed on three identical diets, except for the levels of Se present in each diet. Fresh food was given every 2 days. Mice were maintained for 6 weeks and were weighed once a week prior to being irradiated. Results are the mean weights ± S.E.M. (n=8).

Figure 7.2: Langerhans' Cells ATPase stained in mouse epidermis (x160).



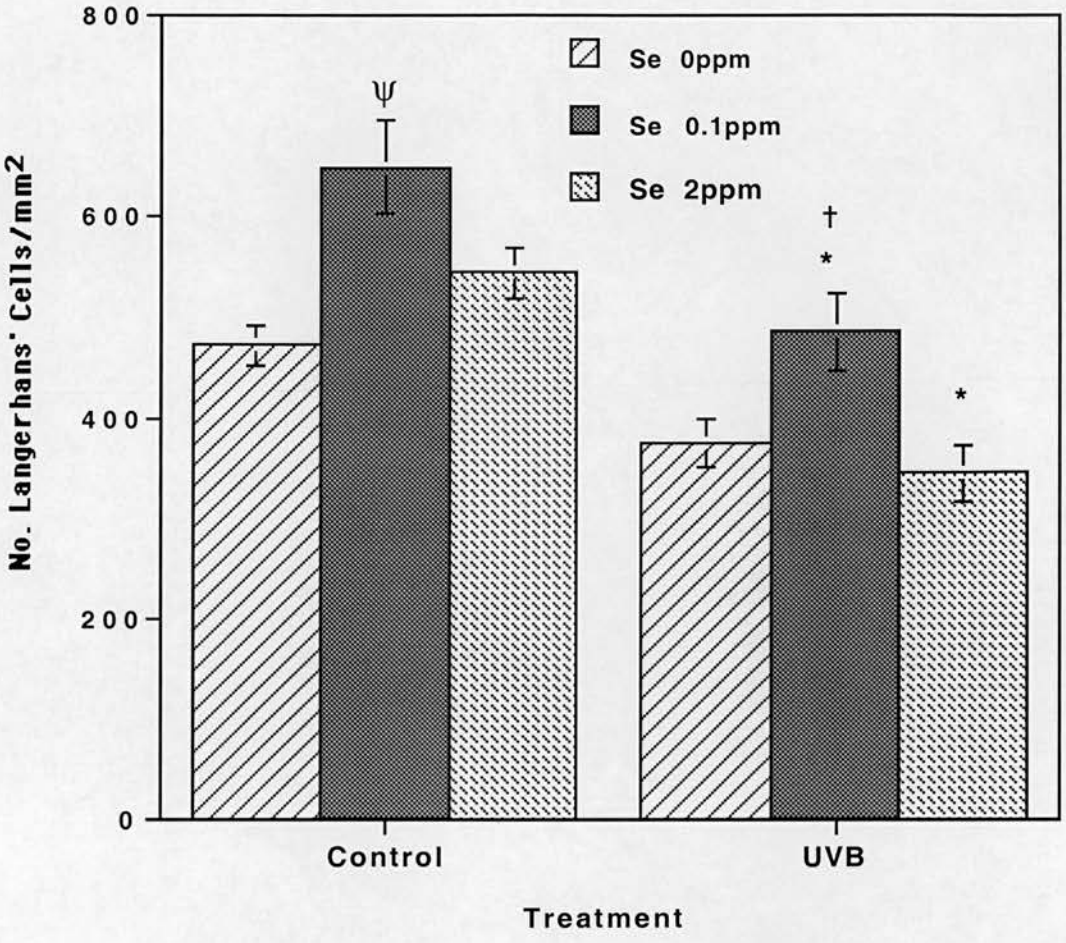
Control mouse epidermis showing ATPase-stained LCs (x160). The LCs stain dark black/brown and can be distinguished by their dendritic appearance.

Figure 7.3a: Effect of level of dietary Se on the numbers of LCs present in the epidermis of C3H/HeN mice, before and after exposure to UVB (Experiment 1).



Groups of mice were maintained on three different diets each with a different level of Se for 6 weeks. The mice were subsequently irradiated or mock irradiated with 1440 J/m^2 UVB. After 24 hours the ears were harvested and ATPase stained for LCs, ten fields from each epidermal sheet were counted for LCs. Results are the mean number of LCs/mm^2 ± S.E.M, n=4. * = Significant decrease in LC number after exposure to UVB compared to control mice, $P<0.05$. † = Significant increase in LC number compared to irradiated, Se-0ppm mice $P<0.05$. φ = Significant increase in LC number compared to control 0ppm mice, $P<0.05$.

Figure 7.3b: Effect of level of dietary Se on the numbers of LCs present in the epidermis of C3H/HeN mice, before and after exposure to UVB (Experiment 2).



Groups of mice were maintained on three different diets each with a different level of Se for 6 weeks. The mice were subsequently irradiated or mock irradiated with 1440 J/m^2 UVB. After 24 hours the ears were harvested and ATPase stained for LCs, ten fields from each epidermal sheet were counted for LCs. Results are the mean number of LCs/mm^2 ± S.E.M, n=4. * = Significant decrease in LC number after exposure to UVB compared to control mice, P<0.05. † = Significant increase in LC number compared to irradiated, Se-0ppm mice P<0.05. φ = Significant increase in LC number compared to control 0ppm mice, P<0.05.

significantly increased numbers of LCs. However, in the second experiment whilst the numbers of LCs present in the mice fed the diets containing 2ppm Se, were higher than in the Se-deficient mice, the increase was not statistically significant (Fig 7.3b).

Following exposure to UVB, there was a decrease in the number of LCs present in the skin, in the mice maintained on the Se-deficient diet and in the mice maintained on the 0.1ppm Se diet (Fig 7.3a & b). In the Se-deficient mice the decrease was around 10%, but this did not achieve statistical significance. In the mice fed the diet containing 0.01ppm Se, the decrease in LCs number following exposure to UVB was significant, at around 40%. There were, however, still significantly more LCs in epidermis of the 0.1ppm fed mice after exposure to UVB, than in the Se-deficient mice. These results were reproducible and each mouse group contained 4 mice and the experiments were carried out three times. In one experiment, the mice fed the diet containing 2ppm Se, had an 11% increase in the number of LCs following exposure to UVB (Fig 7.3a). However, when the experiment was repeated, whilst there were more LCs present prior to irradiation than in the deficient mice, there was a large decrease in the number of LC present following exposure to UVB. The 2ppm Se diet experiments were carried out on groups of 4 mice twice.

7.2.3 Effect of dietary Se on the GPX activity in the mice.

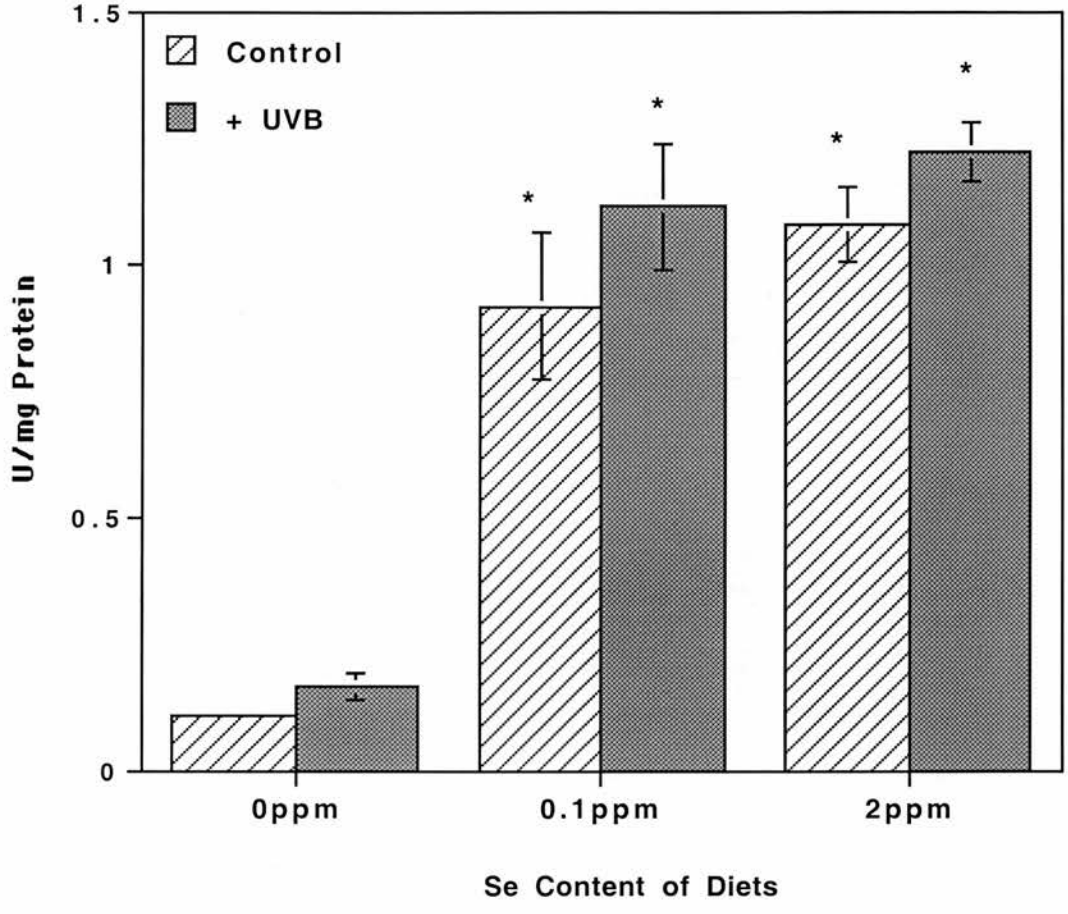
At the end of the 6 week period of maintenance on the Se diets, when the ears were removed for LC counts, the livers, draining lymph nodes and one ear were also harvested and snap frozen in liquid nitrogen. The level of activity for GPX was measured in these frozen tissues. The measurements of activity of GPX, were normalised using the protein measurements (Full methodology for the GPX measurements and the Bradford protein assay are in Chapter 2 section 2.7.4 and 2.7.5).

The activities of GPX in the livers of the mice varied with the concentration of Se in the diet. Selenium-deficient mice had GPX levels that were only 10% of that seen in Se replete mice. Mice fed the 2ppm Se diet, had GPX levels 5% higher than the mice fed on the 0.1ppm Se diet. UVB had no effect on the activity of GPX in the livers (Fig 7.4).

The activity of GPX in the skin of Se-deficient mice was 57% lower ,than the GPX activities in the skin of the mice fed on the 0.1ppm diet. There was a slight decrease in GPX levels in the mice fed on the 2ppm diet, compared to those fed the 0.1ppm diet but this was under 10% and not significant. Again UVB had no effect on the activity of GPX in the skin (Fig 7.5).

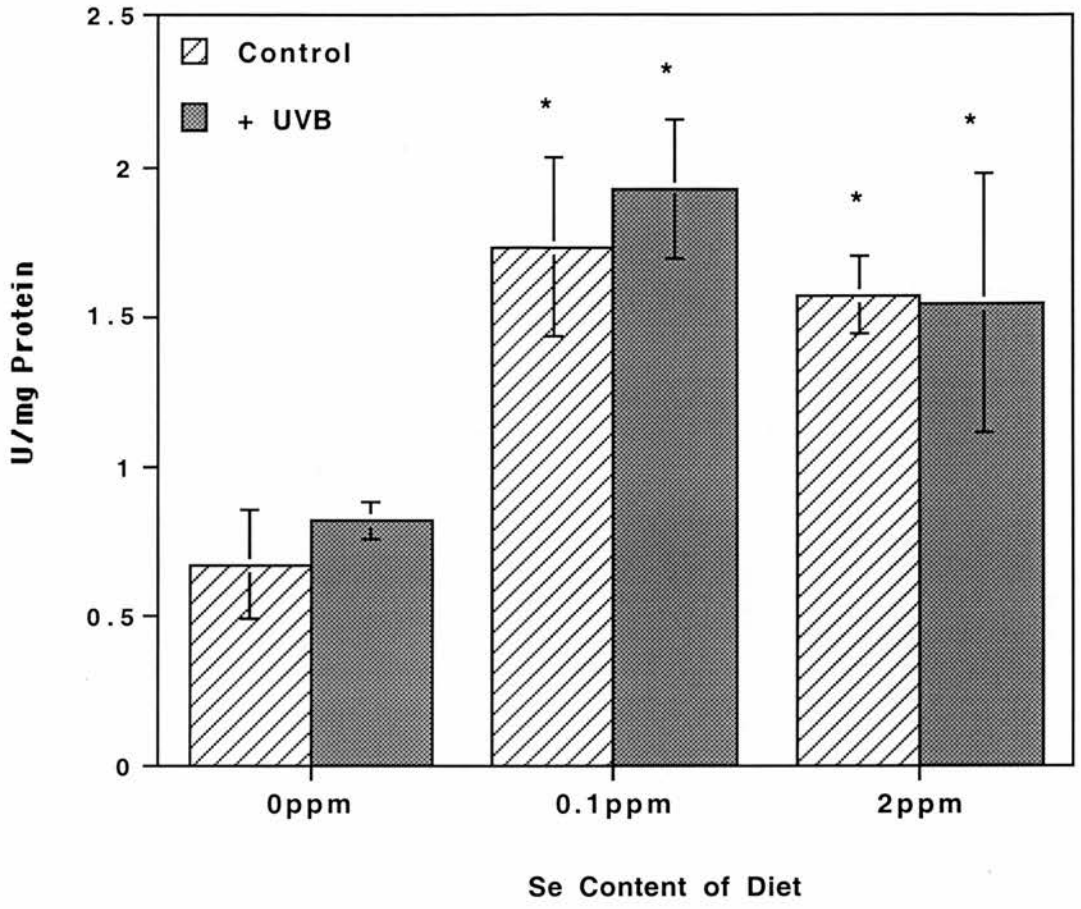
Finally GPX activity in the lymph nodes was significantly lower (47%), in the Se-deficient mice, compared to the mice fed on the 0.1ppm Se diet. Again the GPX activities in mice fed either the 0.1ppm or the 2ppm diets, were not significantly different. UVB did not affect the activity of GPX in the lymph nodes (Fig 7.6).

Figure 7.4: Effect of dietary Se on the activity of GPX in the livers of C3H/HeN mice.



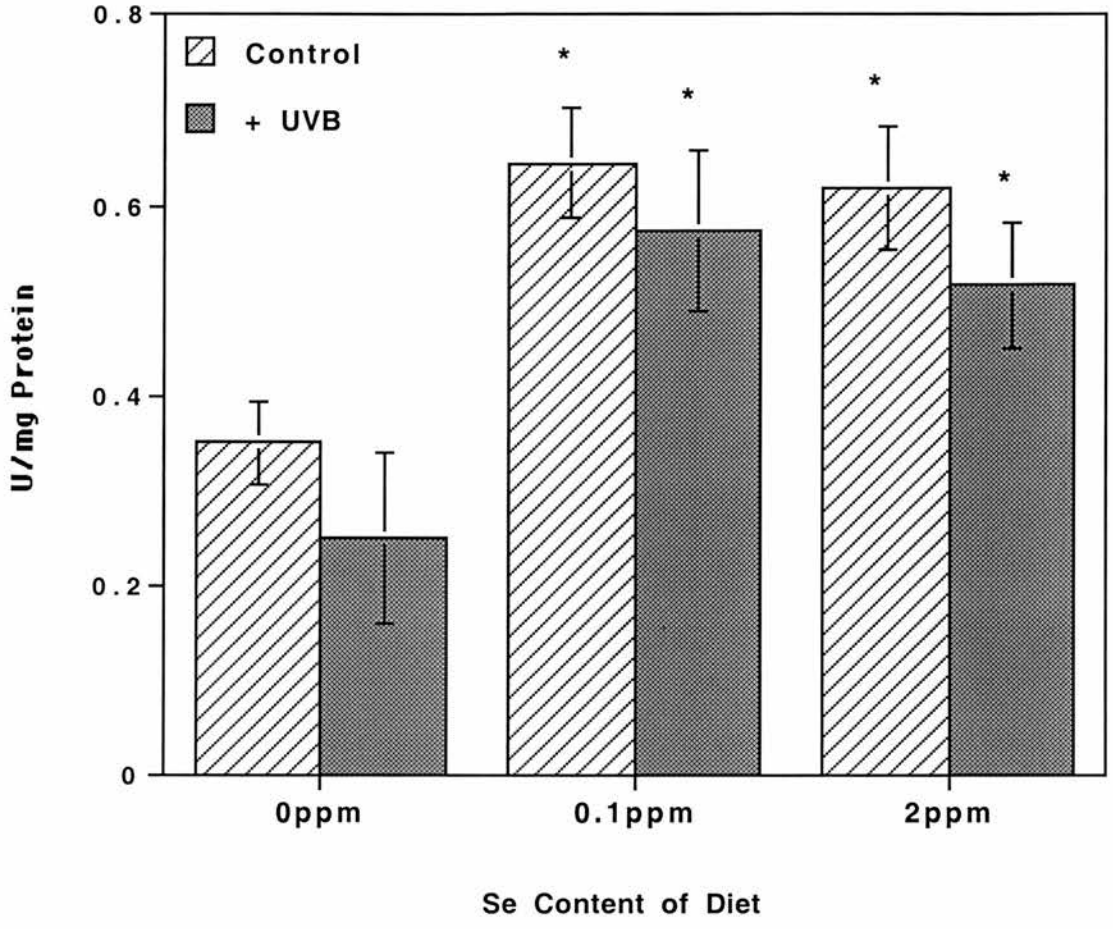
Mice were maintained on one of three diets each with a different level of Se for 6 weeks. They were then irradiated with 1440 J/m^2 UVB or mock irradiated. After 24 hours the livers were harvested and the activity of GPX measured. Results are the mean units/g protein ± S.E.M, n=4. * = Significant difference from Se-deficient control mice, $P<0.05$.

Figure 7.5: Effect of dietary Se on the activity of GPX in the skin of C3H/HeN mice.



Mice were maintained on one of three diets each with a different level of Se for 6 weeks. They were then irradiated with 1440 J/m^2 UVB or mock irradiated. After 24 hours the ears were harvested and the activity of GPX measured. Results are the mean units/g protein ± S.E.M, n=4. * = Significant difference from Se-deficient control mice, $P<0.05$.

Figure 7.6: Effect of dietary Se on the activity of GPX in the lymph nodes of C3H/HeN mice.



Mice were maintained on three diets each with a different level of Se for 6 weeks. They were then irradiated with 1440 J/m^2 UVB or mock irradiated. After 24 hours the lymph nodes were harvested and the activity of GPX measured. Results are the mean units/g protein ± S.E.M, n=4. * = Significant difference from Se-deficient control mice, $P<0.05$.

7.3 Discussion.

Dietary Se is essential for many functions in the body, including the immune system. Selenium can boost B cells, T cells, natural killer and cytotoxic T cells functions (Spallholz *et al*, 1973, 1975, 1990; Meeker *et al*, 1985; Kiremidjian-Schumacher *et al*, 1992,1996; Petrie *et al*, 1989a and b; Koller *et al*, 1986). Whilst a considerable amount of research has been done to investigate the importance of Se on the immune system, as yet no work has been carried out to study the effect of Se on antigen presentation or on LC numbers or function. In the present study, it was demonstrated that Se can influence LC populations in the skin (Fig 7.3a & b). The skin is the organ most likely to encounter foreign antigens. Therefore it is essential that it is equipped to mount a full and competent immune response to them. It would appear that a diet containing at least 0.1ppm Se, was the optimum level of Se for maintaining maximal numbers of LCs in the epidermis in mice (Fig 7.3a & b). In mice maintained with the 2ppm Se diet, there is also an elevated level of epidermal LCs.

There is mounting evidence, to suggest that LCs are very sensitive to damage caused by exposure to free-radicals (Horio and Okamoto, 1987; Iwai *et al*, 1999). It could be argued, therefore, that in Se-deficiency there would be a decrease in GPX and other selenoenzymes with antioxidant functions. The decrease in antioxidant selenoenzymes, would increase the susceptibility of LCs to free radical attack.

Mice fed the diets with different Se content showed no significant difference in weight gain over the 6 weeks of the experiment, suggesting that there was no Se toxicity caused by any of the diets (Fig 7.1b). The Se diets were amino acid based and initial experiments showed that animals fed the Se diets gained weight, at the same rate as the mice fed on the usual commercial diet (7.1a).

Following exposure to UVB, the number of LCs present in the skin decreases (Toews *et al*, 1980). It is important to maintain the LC population in the skin following exposure to UVB, as LCs are required to mount an immune response to any altered self-antigens caused by exposure to UVB. Selenium proved to be important in maintaining the number of LCs present in the

epidermis following exposure to UVB (Fig 7.3a & b). It was found that mice fed the 0.1ppm Se containing diet retained higher numbers of LCs, compared to the Se-deficient mice following exposure to UVB.

If immune surveillance is decreased following exposure to UVB, this may lead to an increased risk of tumour cells not being eliminated by the immune system. This has been shown to occur by Kripke *et al* (Fisher and Kripke, 1976; Kripke, 1977; Kripke, 1984). This group have demonstrated that skin cancers are highly immunogenic, however if a tumour is transplanted into an irradiated host the tumour can grow, as the UVB induces immune suppression in the host. This immunosuppression allows the tumour to evade the host immune response. The immune suppression caused by UVB also causes the body not to respond to other antigens, such as chemicals and microbes. Therefore if the body is to prevent tumour growth and infections, it is essential that antigen presenting ability is not compromised in the skin. The observation that in mice the number of skin tumours produced by exposure to UVB is decreased by Se supplementation, may thus be due, in part, to the maintenance of LC numbers and therefore adequate immune surveillance (Pence *et al*, 1994).

The decrease in LC numbers present in the epidermis following exposure to UVB, is probably accounted for by several mechanisms including: cell death due to free radical damage (Horio and Okamoto, 1987; Iwai *et al*, 1999), DNA damage (Sontag *et al*, 1995) or death by apoptosis (Rattis *et al*, 1998). Furthermore, it may also be due to the LCs binding altered self antigens and moving to the lymph nodes to present them to T cells. One possible mechanism for the protection of LCs from UVB-induced damage may be that, Se decreases the amount of oxidative damage caused by UVB. Other antioxidants can protect the population of LCs present in the skin from free-radical damage induced by UVB. These include; glutathione and SOD, both of which prevent free-radical damage induced by UVB to LCs (Horio and Okamoto, 1987; Iwai *et al*, 1999). These studies also demonstrate that oxidative damage caused by exposure to UVB, is one of the major causes for LC migration and death following irradiation. In Chapter 3 it was demonstrated that lipid peroxidation induced by exposure to UVB is diminished in epidermal keratinocytes, if they are cultured in the presence of Se. Therefore, if the free-radical stress to LCs could be decreased by the

presence of Se, this may help to protect them. Similarly the level of apoptotic cell death (Chapter 4) and oxidative DNA damage (Chapter 5) induced by exposure to UVB, was decreased in keratinocytes by Se supplementation. However, further work needs to be carried out to clarify the mechanism by which Se provides protection against UVB-induced damage. It can be proposed that if there was less oxidative damage induced in the skin, this would lead to less DNA damage and therefore fewer of the cells would undergo cell death by apoptosis. In order to decrease the levels of oxidative damage, Se would have to increase the levels of antioxidant enzymes such as GPX and TR. This is certainly true for the levels of GPX found in the liver, skin and lymph nodes of the mice fed on the diets containing higher levels of Se; the GPX levels were much higher than in the mice fed with the Se-deficient diet (Fig 7.4, 5 and 6). This is of particular importance in the skin where the mice fed the Se replete diets displayed GPX levels, which were 57% higher than in the mice fed with the deficient diets. The increases in antioxidant enzyme levels correlate with the increased numbers of LCs. Therefore it may be that LCs which have a higher level of GPX, have a greater potential to reduce free-radical species and survive exposure to UVB. In the mice fed the Se-replete diets, it would be expected that in addition to increased levels of GPX, the levels of other antioxidant selenoproteins would be increased, such as TR.

The results have clearly shown that in Se-deficiency, the loss of GPX in the liver was considerably greater than that observed in skin and draining lymph nodes. It is likely that the differences represent the different degrees of oxidative stress these tissues are exposed to. The skin is continually exposed to free radicals induced by exposure to sunlight, pathogens and chemicals. Thus it may be that the skin and lymph nodes are high in the hierarchy of tissues for Se retention in times of Se-deficiency. Other organs which show little change in cGPX activity in Se-deficiency are the brain and endocrine tissue organs which are subjected to high and continual levels of oxidative stress (Arthur *et al*, 1993; Beckett *et al*, 1993; Bermano *et al*, 1995; Thompson *et al*, 1995).

Exposure to UVB did not decrease the levels of GPX in the skin. Although only one time point was studied, the results are in agreement with findings in the literature, where at no time after exposure to UVB do the levels of GPX

decrease in the skin (Pence and Naylor, 1990; Shindo *et al*, 1994; Fuchs *et al*, 1989a and b). Many other antioxidant enzymes decrease in expression following exposure to UVB, these include SOD and catalase. It maybe that GPX has a shorter half-life than these other antioxidants, so the levels do not drop.

The migration of LCs out of the epidermis following exposure to UVB, is due in part, to the production of TNF-α by keratinocytes (Cumberbatch *et al*, 1994, Moodycliffe *et al*, 1994; Cumberbatch and Kimber, 1995). UVB induces the production of TNF-α from both human and mouse keratinocytes (Kock *et al*, 1990). Moreover, the induction of mRNA for TNF-α in both mouse and human keratinocytes, and the induction of the protein in human keratinocytes, by exposure to UVB, was demonstrated in Chapter 6 (Figs 6.5 and 6.16). In Chapter 6 the UVB-induction of TNF-α mRNA in human keratinocytes and the protein for TNF-α were decreased by Se supplementation (Fig 6.10 and 6.16). In mouse keratinocytes the TNF-α mRNA was decreased, although the TNF-α protein was not detectable by ELISA. However, if the protein abundance for TNF-α is decreased by Se, this could be an explanation for the preservation of the numbers of LCs in the epidermis.

7.4 Summary.

The results presented in this chapter suggest that Se plays a role in maintaining the population of LCs in the epidermis. This is of great importance to the body, as the skin is continually under assault from pathogens, sunlight and chemicals. Accordingly, the skin must have an efficient system for the presentation of antigens to the immune system. Also UVB induces immune suppression in the skin, one mechanism by which it does this is by loss of epidermal LCs. However in this study, it has been shown that Se can help to maintain a higher population of LCs in the skin following exposure to UVB. This would aid the body in dealing with the carcinogenic effects of UVB, pathogens and chemicals.

7.5 Further work.

Many experiments could be carried out to clarify how Se is acting in the mouse epidermis. Further studies could be performed on the levels of other selenoprotein levels in the skin following Se supplementation, including TR and the individual members of the GPX family. Whether Se decreases the levels of free radicals found in the mouse epidermis could be investigated. Following UVB exposure, experiments could be carried out to measure the effect of Se on DNA damage, apoptosis, and TNF α production in the epidermis. Finally the LCs themselves could be investigated, to see if their antigen presenting ability is modulated following Se supplementation.

Chapter 8

Selenoprotein Expression In The Skin

8.1 Introduction.

The skin contains many enzyme systems to prevent free radical-induced damage including; catalase, SOD and the Se-containing GPX and TR. Catalase and SOD activity are decreased following UVB exposure, whilst GPX appears to be resistant to the deleterious effects of UVB (Shindo *et al*, 1994).

There is much evidence to suggest that Se has an important role in protecting skin from the harmful effects of UVB, this evidence is discussed fully in Chapters 1 and 3. However briefly, in mice Se supplementation can substantially decrease the amount of skin damage, tumour formation and overall mortality following exposure to UVB (Pence *et al*, 1994; Stewart *et al*, 1996). Furthermore, cultured skin cells become resistant to UVB damage following exposure to Se (Rafferty *et al*, 1998).

All the actions of Se were once thought to be exerted through cGPX, but it is now recognised and accepted that Se exerts many of its effects through the expression of a number of selenoproteins. These selenoproteins have Se covalently linked within the protein, as selenocysteine (Cone *et al*, 1976). At least 30 selenoproteins have been identified by SDS-polyacrylamide electrophoresis of [^{75}Se]-labelled tissue. However, only approximately fourteen of the detected proteins have been characterised, including cGPX, PHGPX, EGPX, selenoprotein-P, SP56, protein disulphide isomerase, TR and the iodothyronine deiodinases (IDI) (Arthur *et al*, 1996, Burk and Hill, 1993). The effects of UVB on the expression of these selenoproteins, or indeed their precise roles in controlling the cells response to UVB are not known. The family of GPX proteins detoxify a wide range of lipid peroxides (Rotruck *et al*, 1973). These enzymes have been implicated in protecting cells from UVB-induced free radical damage (Moysan *et al*, 1995). TR is a flavin adenine dinucleotide-containing enzyme found in all organisms. In conjunction with it's substrate thioredoxin, TR forms a redox system which has multiple

functions including detoxification (Holmgren and Bjornstedt, 1995; Tamara and Stadtman, 1996; Gladyshev *et al*, 1996). The characteristics of the individual selenoproteins are discussed in detail in Chapter 2. The molecular weights of a selection of selenoproteins can be found in Table 8.1.

Table 8.1: Molecular weights of the best known selenoproteins.

Selenoprotein	Molecular Mass (kDa)	Function
PHGPX	19-21	Antioxidant
TR (3 isoforms)	57-60	Antioxidant
cGPX	22-23	Antioxidant
Type 1 IDI	27	Thyroid hormone metabolism
Selenoprotein-P	42	Antioxidant, Se transport protein
14 kDa Protein	14	Unknown
Selenoprotein-W	10	Muscle protein

The expression of most selenoproteins is regulated by the bioavailability of Se (reviewed in Allan, 1999). These include PHGPX, cGPX, Selenoprotein-P and TR. It has been demonstrated that in most tissues, PHGPX activities are well conserved in Se deficiency, whilst in contrast cGPX is quickly lost (Weitzel *et al*, 1990).

Thus, PHGPX is preferentially retained at the expense of cGPX during periods of Se-deficiency (Weitzel *et al*, 1990). PHGPX is thought to be very important in protecting the cellular membrane as it reduces lipid peroxides (Ursini *et al*, 1985). Whilst cGPX is thought to react with soluble hydroperoxides. It was also found that the levels of TR in many tissues including the brain appear to be conserved during Se deficiency (Hill *et al*, 1997).

Labelling experiments with [^{75}Se] have shown clear tissue differences in the pattern of selenoprotein expression (Behne *et al*, 1988), but studies have not been performed on human skin. Such studies may indicate which

selenoproteins could have a role in protecting skin from the harmful effects of UVB.

In the epidermis, keratinocytes normally move up through the epidermis, differentiate and eventually form the cornified envelope of the skin. In culture, keratinocytes are maintained in low levels of calcium to prevent differentiation. However when calcium is added to the media the keratinocytes will begin to differentiate. The cells can be seen to become enlarged and more rectangular, with some fragments of cornified material present in the media. There are reports, which suggest that whilst undergoing differentiation the activities of intracellular antioxidant enzymes increase. One of the enzymes which has been shown to increase is GPX (Vessey *et al*, 1995). Whereas, TR activity has been shown to diminish when calcium is added to keratinocytes (Schallreuter *et al*, 1986b). Therefore the effect of calcium on the selenoprotein profile of primary keratinocytes was studied.

The aims of this sudy were to:

- To define the profiles of selenoproteins expressed by cultured human keratinocytes, melanocytes and fibroblasts.

- Compare the selenoprotein profiles of primary keratinocytes to that found in epithelial derived cell lines.

- To investigate the effect of exposure to UVB on the selenoprotein profiles expressed by primary skin cells. Also to study the selenoprotein profile in differentiating primary keratinocytes.

- Confirm that patients plasma Se levels increase following Se supplementation.

8.2 Methods and Results.

8.2.1 Selenoprotein profiles of skin cells.

Primary keratinocytes, melanocytes and fibroblasts were grown until 70% confluent, in large 75 cm^2 tissue culture flasks. They were then labelled for 72 hours with [^{75}Se]-selenite (0.02 MBq/ml). Cells were harvested and the proteins were then separated by SDS-PAGE and visualised as described in full in Chapter 2, section 2.8.1.

Primary keratinocytes, melanocytes and fibroblasts were found to have clearly different selenoprotein profiles (Figs 8.1 and 8.2). Each cell type showed up to 10 major selenoproteins (molecular mass ranging from approximately 10 to 60 kDa), but many minor selenoproteins were also found to be expressed. It is of particular note that between cell types there was variable expression of the 21 kDa and 60 kDa selenoproteins. Western blotting identified the 21 kDa and 60 kDa selenoproteins as PHGPX and TR respectively (This work was carried out by Dr John Arthur, Rowett Research Institute, Aberdeen) (Rafferty *et al*, 1998). The 60 kDa [^{75}Se] labelled bands in each cell line, corresponded exactly in mobility and relative intensity to the bands revealed by western blotting. The western blot was not sensitive enough to detect the PHGPX directly, however, a standard of pure PHGPX had the same mobility as the 21 kDa [^{75}Se]-labelled band. The greatest expression of TR and PHGPX was found in fibroblasts, with keratinocytes expressing relatively little of these selenoproteins. The rank order of PHGPX and TR expression was fibroblasts>melanocytes>keratinocytes.

The selenoprotein bands common to all cell types investigated, were of the following masses a 60 kDa band, which was identified as TR. The 21 kDa, which was identified as PHGPX and bands at 27 kDa, 23 kDa, 18 kDa, 16 kDa, and finally 14 kDa which were unknown. A band at 17 kDa was expressed to a high level in the fibroblasts and was expressed weakly in the keratinocytes.

The levels of selenoproteins expressed by other epithelial cell lines were also investigated. The cell types used were labelled with [^{75}Se]-selenite and separated in the same manner as described for the primary cells. It was

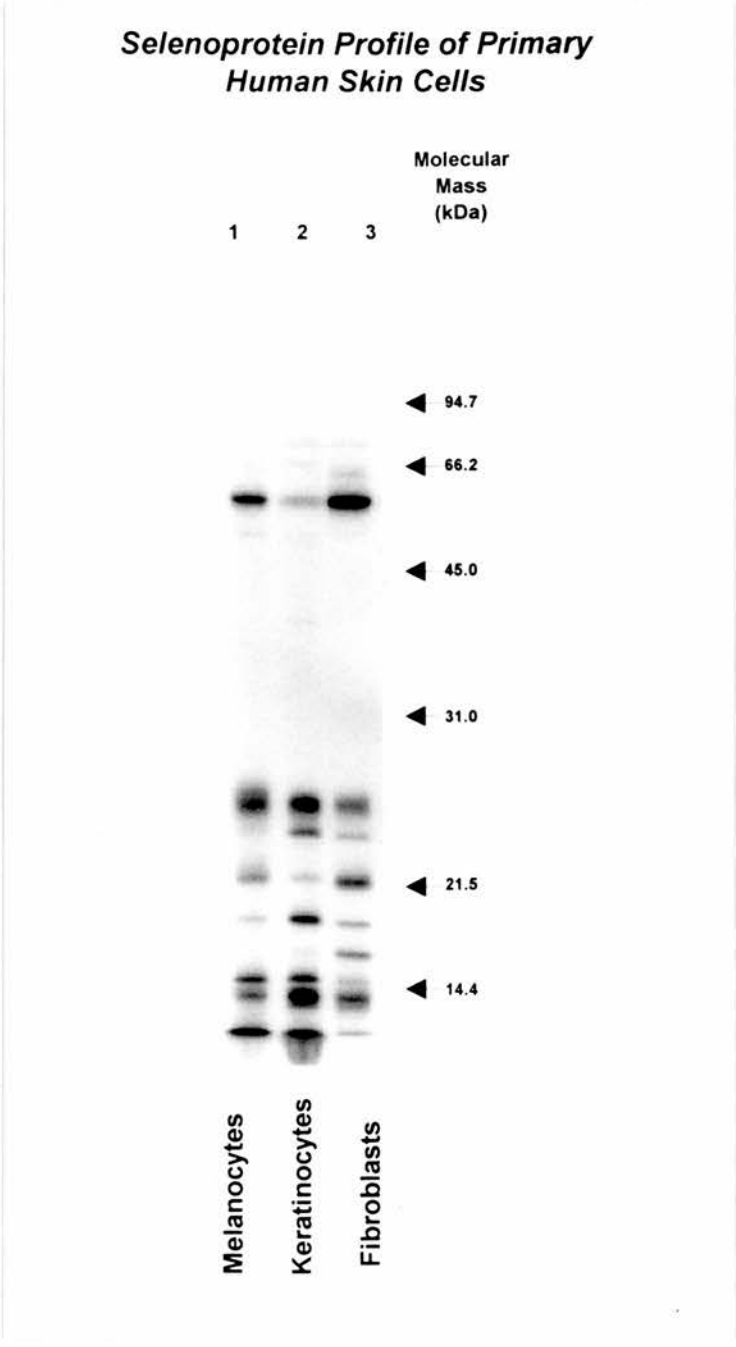
found that the epithelial cell lines tested, had quite distinct selenoprotein profiles when compared to primary keratinocytes (Fig 8.3 and 8.4). SiHa cells (derived from a squamous cell carcinoma of the uterus) (Friedl *et al*, 1970) expressed higher levels of TR and lower levels of the other lower molecular weight selenoproteins, when compared to primary keratinocytes. A431 cells (human epithelial carcinoma cell line) (Giard *et al*, 1973) were very similar to primary keratinocytes in their selenoprotein profile, however they did appear to express less of the 30 and 23 kDa selenoproteins. The selenoprotein profile in HeLa cells (derived from a carcinoma of the cervix) (Scherer *et al*, 1953) was similar to the SiHa cells, in that they overexpressed TR and exhibited a decreased expression of the lower molecular weight selenoproteins when compared to primary keratinocytes. Finally, the HaCaT cells (spontaneously transformed human keratinocyte line) (Boukamp *et al*, 1988) expressed more TR and PHGPX than the primary keratinocytes.

The primary skin cells were also cultured in 10cm petri dishes and labelled with [^{75}Se] for 24 hours, prior to the media being replaced with PBS and the cells exposed to 100 J/m^2 UVB. The original [^{75}Se]-containing media was then replaced and the cells incubated for a further 24 hours, before being harvested and the selenoproteins separated.

Exposure to UVB did not appear to alter the expression of any of the selenoproteins present (Fig 8.5).

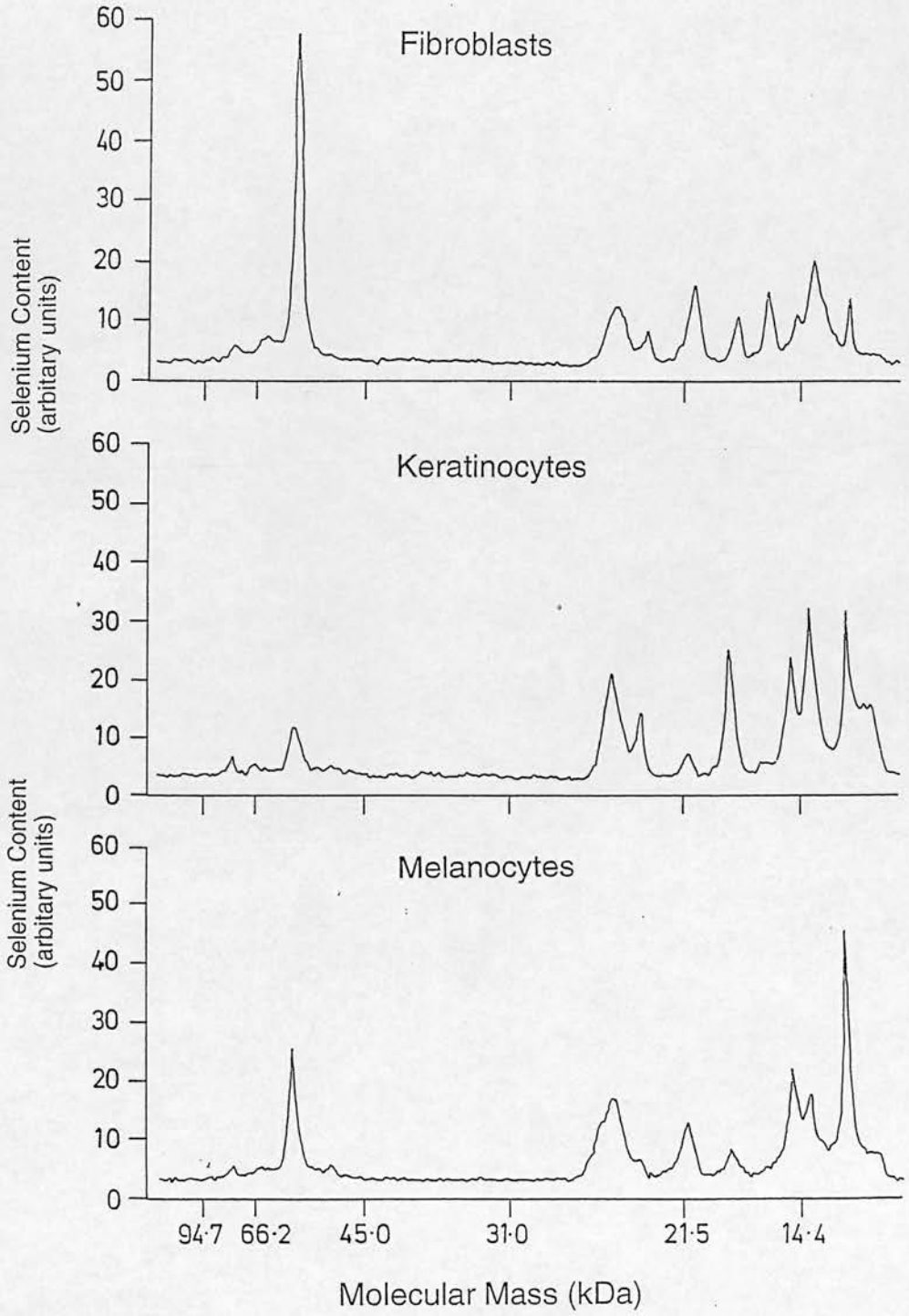
Keratinocytes were treated with 1.5 mM calcium chloride to induce cellular differentiation. The cells were treated with calcium chloride for 24 hours, prior to being labelled with [^{75}Se] for a further 48 hours, in the presence of 1.5 mM calcium chloride. The selenoprotein profiles in differentiating keratinocytes was very similar to that of normal keratinocytes, except that the protein for TR appears to show a slight decrease in expression.

Figure 8.1: Selenoprotein expression by primary human skin cells.



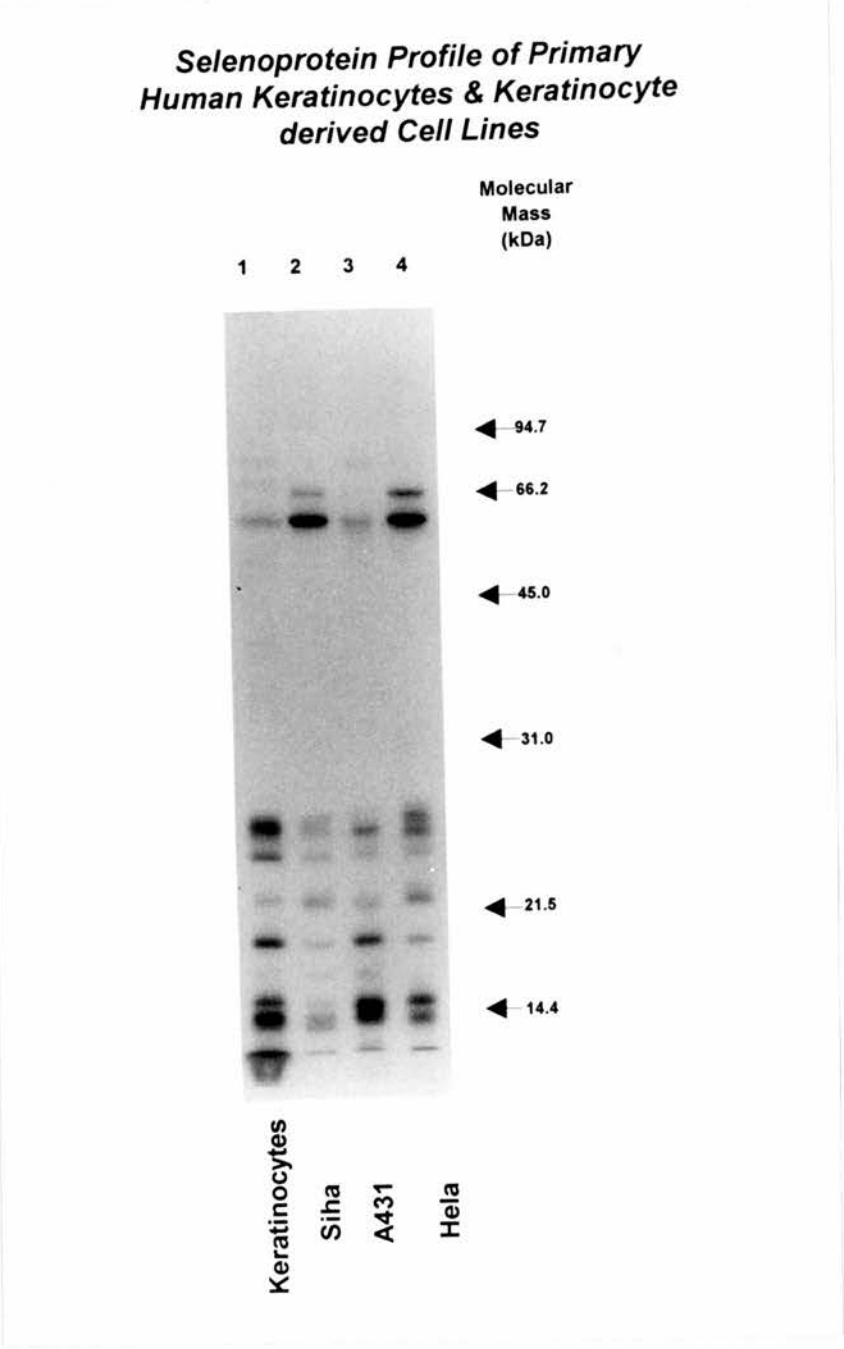
Lane 1 = Melanocytes; Lane 2 = Keratinocytes; Lane 3 = Fibroblasts. Cells were labelled with [^{75}Se]-selenite for 72 hours and the proteins were separated by SDS-PAGE and visualised by autoradiography.

Figure 8.2: Selenoprotein profile analysis of primary human skin cells.



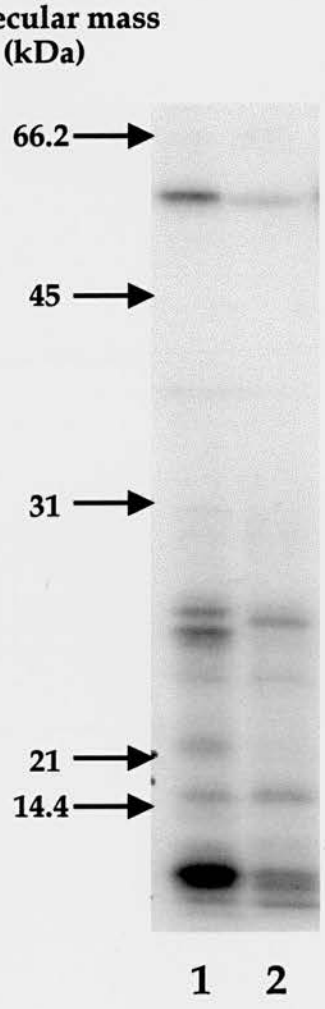
A phosphoimager was used to quantify the radioactivity of the selenoproteins expressed by the primary skin cells. The protein gels were scanned overnight on a phosphoimager and the profiles of the selenium-containing proteins were generated. The molecular masses are shown on the bottom profile.

Figure 8.3: Selenoprotein expression by primary human keratinocytes compared to epithelial derived cell lines.



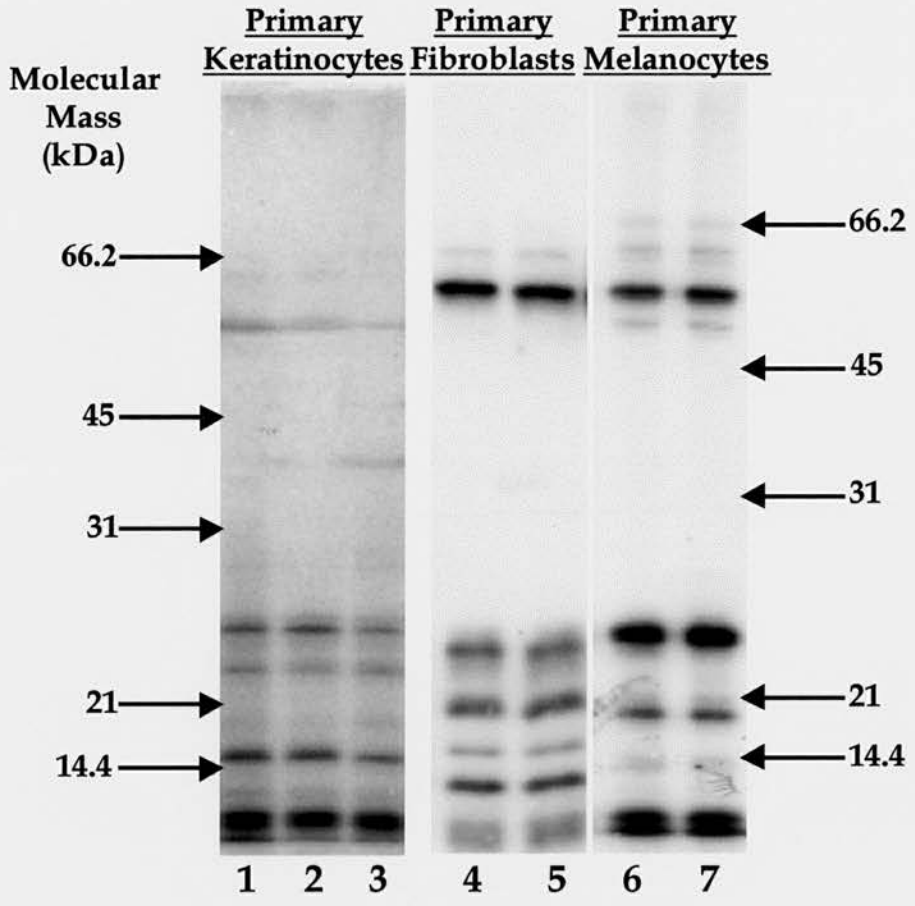
Lane 1 = Primary human keratinocytes; Lane 2 = SiHa cells; Lane 3 = A431 cells; Lane 4 = HeLa cells. Cells were labelled with [^{75}Se]-selenite for 72 hours and the proteins were separated by SDS-PAGE and visualised by autoradiography.

Figure 8.4: Selenoprotein expression in primary human keratinocytes compared to HaCat cells.



Lane 1= HaCaT cells; Lane 2 = Primary human keratinocytes. Cells were labelled with [^{75}Se]-selenite for 72 hours and the proteins were separated by SDS-PAGE and visualised by autoradiography.

Figure 8.5 Selenoprotein expression in primary skin cells exposed to UVB radiation.



Lane 1= keratinocytes; Lane 2 = keratinocytes exposed to 100 J/m^2; Lane 3 = keratinocytes treated with 1.5 mM calcium chloride, Lane 4 = fibroblasts; Lane 5 = fibroblasts exposed to 100 J/m^2; Lane 6 = melanocytes; Lane 7 = melanocytes exposed to 100 J/m^2. Cells were labelled with [^{75}Se]-selenite for 24 hours prior to exposure to 100 J/m^2 UVB, after exposure the original [^{75}Se]-containing media was returned to the cells and they were incubated for a further 24 hours. The cells were then harvested and the proteins separated by SDS-PAGE and visualised by autoradiography. The keratinocytes treated with 1.5 mM calcium chloride were incubated with the calcium chloride for 24 hours prior to being labelled with [^{75}Se] for 48 hours (during the 48 hours labelling the calcium chloride also present).

8.2.2 Selenium content of culture media, plasma and skin.

Selenium measurements were carried out by acid digestion and fluorimetry, the full method can be found in Chapter 2, section 2.2.5.

Selenium content of tissue culture media.

All the culture media used in this project were analysed for their Se content:

Table 8.2: Selenium content of tissue culture media.

Media	Selenium Content (nM)
Primary Keratinocyte	8.9
Primary Melanocyte	28
DMEM 5% FCS	0
DMEM 10% FCS	44.7

The tissue culture media contained either very low levels of Se or was too low to be detected (Table 8.2). Different batches of FCS also demonstrated variations in Se content (Table 8.2).

The effect of Se supplementation on human plasma Se levels.

A small trial (n=6) with patients was also carried to determine if plasma Se levels could be increased following Se supplementation. The patients were part of a larger clinical trial being carried out in the Department of Dermatology, University of Edinburgh, UK. The patients received 400 μg of sodium selenite in capsule form, daily for 6 weeks.

The average selenium content of the plasma for these patients prior to Se supplementation was found to be 89.33 ng/ml ±11.8 (S.E.M). After Se supplementation for six weeks, the average Se content increased to 104.7 ng/ml ± 9.5 (S.E.M), this represented a 17.2% increase in plasma Se.

Finally the Se content of human skin was measured. To obtain sufficient material to assay the Se levels, skin from foreskin samples had to be used.

The foreskins were obtained from The Royal Sick Children's Hospital, Edinburgh, UK. The level of Se present in the skin was 84.41 ng/g and 89.11 ng/g for both samples tested.

8.3 Discussion.

The family of GPX proteins and the family of TR proteins, are both clear candidates for selenoproteins which may mediate the protective effects of Se. The glutathione peroxidases can detoxify hydrogen peroxide, lipid hydroperoxides and phospholipid hydroperoxides, which are produced during UVB exposure. Thioredoxin reductase is a selenoenzyme with multiple functions, which can act through its ability to reduce oxidised thioredoxin (Holmgren and Bjornstedt, 1995), or by its ability to directly reduce hydrogen peroxide and lipid hydroperoxides in the presence of NADPH (Bjornstedt *et al*, 1995a). Thus, TR may play an important role in protecting cells from peroxidative damage. However, labelling of tissues with [^{75}Se]-selenite has allowed the detection of more than 30 selenoproteins, with the pattern of selenoprotein expression quite different between tissues. It is thought that some of these selenoproteins may also be involved in detoxification reactions and in modifying cell growth or cell death (Sunde, 1990).

The expression of the 21 and 60 kDa selenoproteins showed the most variability between cell types. These selenoproteins were identified by western blotting as PHGPX and TR respectively. We found that keratinocytes expressed much lower levels of TR and PHGPX, when compared to melanocytes and yet keratinocytes were found to be more resistant to UVB-induced cell death than melanocytes. A UVB dose of 720 J/m^2 was required to produce 80% cell death in melanocytes, whilst 960 J/m^2 was required to produce 80% cell death in keratinocytes (Chapter 3). Furthermore, despite these differences in selenoprotein expression between the two cell types, keratinocytes and melanocytes showed a similar concentration requirement for Se in order to obtain maximal protection from UVB-induced cell death (Chapter 3). Whilst it could be argued that these observations imply that the selenoproteins TR and PHGPX are not involved in protection, this is not necessarily the case. The susceptibility of a cell to UVB-induced damage will depend on the total expression of a wide range of antioxidant enzymes, DNA repair mechanisms and not necessarily to differences in the expression of a single specific selenoprotein. Thus conclusions regarding the protective role of a specific selenoprotein, made by comparison of the susceptibility of different cell types to UVB-induced cell

death may be misleading. Experiments which relate to the susceptibility of a cell to UVB-damage, by modifying the expression of a single selenoprotein within a single cell type, would provide more conclusive data.

The 23 kDa selenoprotein found in all of the primary cell types may represent cGPX and the 14 kDa protein maybe a newly identified selenoprotein protein described by Gladyshev *et al*, 1998. However, these observations need to be confirmed by western blotting. The 23 kDa protein is expressed to a greater degree in keratinocytes than in fibroblasts and indeed melanocytes express it only weakly. The 14 kDa protein was also expressed more strongly in keratinocytes than in melanocytes and fibroblasts. It is known that cGPX is an antioxidant enzyme, furthermore antioxidant properties have been proposed for the 14 kDa selenoprotein. It is possible that the 23 and 14 kDa proteins are also involved in protecting keratinocytes from the harmful effects of UVB radiation.

Primary human keratinocytes only have a short life span in tissue culture, therefore it is very difficult to obtain large populations of these cells. This limitation on the number of times keratinocytes divide in culture, greatly limits the size of the experiments which can be carried out. Therefore, in the hope that it would be possible to use a epithelial derived cell line for some of the larger experiments, the selenoprotein profiles of some cell lines were compared to that of primary keratinocytes. Unfortunately it was demonstrated that none of the carcinoma derived epithelial cell lines or spontaneously transformed human keratinocytes, resembled the selenoprotein expression in primary keratinocyte, sufficiently to allow them to substitute for primary keratinocytes in experiments. Therefore for the majority of this thesis primary human keratinocytes were used. This unfortunately did place some restrictions on the size of some of the experiments performed. Two of the main differences between primary keratinocytes and the cell lines were the higher level of expression of the TR protein and the lower levels of some of the 30-14 kDa selenoproteins. The higher expression of TR found in the cell lines may have lead to them responding differently to UVB than the primary keratinocytes. The cell lines which were closest in selenoprotein expression to primary human keratinocytes were the A431 cell line and the HaCaT cell line. Consequently the HaCaT cell line was utilised in two experiments in this thesis which

required large numbers of cells. During cell survival experiments, following exposure to UVB the HaCaT cell line responded in an identical manner to primary keratinocytes, this may be due to the similarities they share in selenoprotein expression (Chapter 3).

Following exposure to UVB, the activity of some antioxidant enzymes can decrease. Catalase and SOD activity are decreased following UVB exposure, whilst GPX appears to be resistant to these effects, this was measured over several time points up to 24 hours following exposure to UVB (Fuchs *et al*, 1989a and b; Pence and Naylor, 1990; Shindo *et al*, 1994). It is thought that GPX has a shorter half-life than other antioxidant enzymes and so does not appear to decrease following exposure to UVB. The expression of all visible selenoproteins was not affected by UVB exposure. This is of interest, however only one time point, 24 hours following exposure to UVB was tested. A time course covering more time points, would yield more informative data on the levels of selenoproteins following exposure to UVB.

It has been reported by Vessey *et al*, 1995 that the activity of antioxidant enzymes increase as primary keratinocytes differentiate (this was followed over 3 days). Calcium chloride was used to induce differentiation of the primary keratinocytes (over 72 hours) and it was found that the selenoprotein profiles were similar to untreated keratinocytes (Fig 8.5). The only difference was a slight decrease in TR expression, this would agree with the literature, which suggests that TR activity can be decreased in keratinocytes by calcium (Schallreuter *et al*, 1986b).

It has been demonstrated that the levels of Se in tissue culture media can vary considerably. The Se content of FCS can also vary greatly, with values from zero to 35 μg/L (Leist *et al*, 1996). Therefore, the level of Se was determined in all of the tissue culture media used in this study. The levels of Se in the media were found to be low. The level of protection achieved and the concentrations of sodium selenite and selenomethionine which provided protection from UVB-induced damage, were similar in all of the cell types, despite the different Se content of each media (results from Chapter 3). Therefore there is a lack of any correlation between the Se contents of the media and the concentrations at which the sodium selenite and selenomethionine provide protection. Furthermore, it would appear that the

Se in the tissue culture media is not readily utilised by the cells, unlike the supplemented sodium selenite and selenomethionine.

An important question which needed to be investigated was; following Se supplementation, does the activity of any selenoproteins increase in cells. It has been shown that TR activity increases, following Se supplementation in the keratinocyte cell line HaCaT (Marcocci *et al*, 1997) . The form of Se used was sodium selenite (40 nM) and the level of TR activity was shown to increase by 50%. Indeed even 20 nM selenite was shown to increase the TR activity significantly. The activity of GPX increased dramatically also when HaCaT cells, were supplemented with 20 nM selenite. The GPX activity reached a plateau with 20 nM sodium selenite. The TR enzyme also reached its maximum activity level at 20 nM (Marcocci *et al*, 1997). It has been reported in hepatocarcinoma and colon-carcinoma derived cells, that concentrations as low as 1 nM sodium selenite can increase GPX activity to its maximal level (Baker *et al*, 1998). Therefore, it has been demonstrated that even at low concentrations of Se supplementation, the activity of selenoproteins can reach a maximal level of activity.

Intake of Se in the United Kingdom has fallen considerably over the past two decades, until the present when the average intake of Se is only approximately 40% of the recommended daily allowance (75 μg/day) (Rayman, 1997). It is possible that a contributing factor to the increase in the incidence of all types of skin cancer in the United Kingdom (Ko *et al*, 1994), may be the decline in Se status. As mentioned in the introduction, the sharp decrease in plasma Se levels is thought to be a consequence of the reduction in imports of North American-grown wheat, which are high in Se. The subsequent increase in imports of European-grown wheat, has introduced the use of a form of wheat which contains a lower concentration of Se. Indeed the plasma levels of Se have decreased from 110 μg/L to 70 μg/L in the period between 1985 to 1994 in the UK (Rayman, 1997). However, it has been demonstrated that human plasma Se can be increased easily following Se supplementation (Varo *et al*, 1988). The successful increase in plasma Se levels have been best studied in Finland, where a program of nation-wide Se supplementation (as sodium selenate) in fertilisers has been on going since 1984. It was also shown that the plasma levels in patients in Edinburgh average 89.33 μg/L, this was slightly higher than the levels found in the

literature for the UK (70 μg/L) (Rayman, 1997). Following Se supplementation for 6 weeks the patients' plasma levels had increased to 104.7 μg/L, thus confirming that plasma Se levels are responsive to Se supplementation. However due to the amount of skin required to investigate Se levels, no information on whether Se supplemented in the diet, can reach the skin and increase the levels of selenoproteins present, could be obtained. However previous work in Chapter 7 using mice showed that the level of dietary Se can modify the level of GPX in the skin of mice. Also other groups have demonstrated that the level of dietary Se does modify the level of GPX and overall Se content of the skin (Burke *et al*, 1992b; Pence *et al*, 1994). Therefore it can be concluded that Se supplements in the diet can increase the levels of selenoproteins in skin.

8.4 Further Work.

A great deal of further work could be carried out in this area. The effect of UVB and cellular differentiation on the individual selenoproteins could be further characterised. When antibodies for more of the selenoproteins become available, the presence of greater numbers of selenoproteins expressed by primary skin cells could be confirmed. If an improvement can be made on the sensitivity of the assays used to investigate selenoprotein activity, more detailed investigations into the effect of Se supplementation, on the activity of selenoproteins in primary skin cells could be undertaken. It would be of interest also to attempt to determine the form of Se present in tissue culture media. Individual selenoprotein DNAs could be transfected into skin cells or specifically knocked out, using antisense probes to ascertain their role in protecting cells from UVB. Finally, it would be of interest to study further which selenoproteins increase in the skin following Se supplementation, for example TR and PHGPX have not yet been widely studied.

Chapter 9

Overview

Skin cancer is one of the most common malignancies reported in the UK. The number of cases reported each year has nearly doubled between 1980-1990, for all three main types of skin cancer (Devessa *et al*, 1995). Subsequently, the number of skin cancer related deaths has risen by around 50% over the last 15 years. The number of cases of BCCs are increasing at a rate of 5% per annum. The number of cases of malignant melanoma are increasing at a rate of 7% per annum. The major causative factors leading to the increased number of cases of skin cancer are thought to be; an increase in the number of people taking sunshine holidays and the decrease in the ozone layer, resulting in an increase in the amount UV radiation reaching ground level. The use of sunscreens which filter UVB but not UVA radiation, may also contribute to skin damage. Another potential factor resulting in the rise in skin malignancies, may be the low dietary intake of Se in the UK. The dietary intake of Se in the early 1970's was approximately 60 μg/day, however the current dietary intake of Se in the UK is only approximately 35-40 μg/day, an intake which falls well below the recommended intake of 70 μg/day (Rayman, 1997). Subnormal Se status is associated with a four-fold increased risk of developing skin cancer in humans, and a poorer prognosis in patients with skin cancer (Reinhold *et al*, 1989; Combs *et al*, 1993; Deffaunt *et al*, 1994).

Previous reports concerning the role of Se in the skin, have highlighted the need for a greater understanding of the mechanisms of Se action. The first key studies on the role of Se in the skin, reported that mice supplemented with Se develop less skin damage, skin tumours and have a lower mortality rate, than Se-deficient mice following exposure to UVB (Thorling *et al*, 1983; Overvad *et al*, 1985; Pence *et al*, 1994). Subsequently, it was reported that Se supplementation in humans could decrease the UVB-induced erythema and formation of sunburn cells in the epidermis (Burke *et al*, 1992a and b; LaRuche and Cesarini, 1991).

The work presented in this thesis, attempts to investigate the modulatory effects of Se on various types of UVB-induced damage in the skin. The starting point was to study the gross effects of UV on skin cells. Cells which have been heavily irradiated die by either necrosis or apoptosis. Necrotic cell death occurs at higher levels of UV radiation than apoptosis. It was found that Se supplementation with sodium selenite, at concentrations between 1-200 nM or selenomethionine at concentrations between 10 nM-1 μM protected primary human keratinocytes, melanocytes and fibroblasts from UVB-induced cell death (Fig 3.3a, Table 3.4, Fig 3.5). Cell death induced by UVB in the experiments described in Chapter 3, was concluded to occur mainly by necrosis as high levels of UVB were used. It has been reported that Se supplementation of primary human fibroblasts, decreases the cell death induced by exposure to UVB (Richard *et al*, 1990) and UVA (Leccia *et al*, 1993; Moysan *et al*, 1995). Furthermore, transfection of a gene coding for a selenoprotein with GPX-like activity, into HaCaT cells provides resistance to UVB-induced cell death (Shisler *et al*, 1998). The protective effect of the GPX gene suggests that ROS are involved in the induction of cell death in skin cells exposed to UVB.

Following the discovery that Se supplementation protects cells from UVB-induced cell death, the mechanisms behind this protection were investigated. It was found that protection was not a consequence of Se increasing the growth rate of the cells (Fig 3.1a and Table 3.1a and Table 3.3). It was also found that a 24 hour pre-incubation with Se, was required prior to exposure to UVB to provide significant protection, suggesting that Se acts through increased expression of selenoproteins (Fig 3.8). The protection provided by Se supplementation was not due to the Se compounds acting as sunscreens, as they did not absorb significantly in the UVA and B wavelengths (Fig 3.7).

Many selenoproteins act as antioxidant enzymes. Therefore, the role of Se in protecting cells from oxidative damage was investigated. Oxidative damage was induced by treating cells with menadione. Pre-treatment of primary human keratinocytes with sodium selenite at concentrations ranging between 1-100 nM and selenomethionine at a concentration of 50 nM, protected cells from the oxidative damage induced by exposure to menadione (Fig 3.13). To further investigate the mechanism by which Se protects from UVB-damage, lipid peroxidation was studied. Exposure to

UVB radiation produces ROS, which subsequently lead to oxidative reactions in the cellular membranes, resulting in membrane degradation and cell death. Sodium selenite and selenomethionine, at concentrations between 1-50 nM and 50-200 nM respectively, significantly decreased the UVB-induced formation of lipid peroxides in HaCaT cells (Fig 3.16). In the literature, dietary Se supplementation has been found to decrease the level of UVB-induced lipid peroxidation in humans (Pietschmann *et al*, 1992). Furthermore, the decrease in the formation of lipid peroxides in the presence of Se supplementation, has also been shown with primary fibroblasts following exposure to UVA (Moysan *et al*, 1995; Leccia *et al*, 1993).

Prevention of oxidative damage induced by exposure to UVB radiation, may thus be an important mechanism by which Se increases cell survival following exposure to UVB. The mechanism by which Se prevents lipid peroxidation and menadione-induced damage is likely to be through increased expression of antioxidant selenoproteins. Examples of such selenoproteins are; PHGPX which can reduce lipid peroxides, or TR an enzyme which is involved in the regeneration of ascorbate, which can in turn regenerate α-tocopherol, a lipid peroxidation chain breaking antioxidant. TR may also react with superoxide and hydrogen peroxide and so could decrease the levels of oxidative stress following exposure to UVB.

Following exposure to UVB, apoptosis also occurs. In the skin exposure to UVB causes the formation of apoptotic cells (called sunburn cells), which have a condensed, vacuolated nucleus and a vacuolar cytoplasm (Young, 1987). In the literature, Se supplementation is reported to decrease the UVB-induced formation of sunburn cells in human skin (LaRuche and Cesarini, 1991). Sodium selenite and selenomethionine at concentrations ranging from 10 nM-1 μM and 50 nM-1 μM respectively, decreased the level of UVB-induced apoptosis in primary human keratinocytes (Fig 4.4).

To investigate how Se diminished UVB-induced apoptosis, two of the main pathways of apoptosis were investigated. The first pathway was the p53 pathway. Oxidative stress has been shown to be involved in the activation of p53 protein (Renzing *et al*, 1996). However, it was found that neither sodium selenite or selenomethionine decreased the UVB-induction of the p53 protein (Fig 4.8 and 4.9). The p53 protein appears to accumulate

following exposure to UVB, as a consequence of stabilisation of the p53 protein. In order for stabilisation to occur, the p53 protein must be phosphorylated to prevent the destabilising mdm-2 protein from binding to it (Midgley and Lane, 1997; Sheih *et al*, 1997). Following stabilisation of p53, it must then be phosphorylated again to increase its ability to bind DNA and activate downstream genes (Lu *et al*, 1998). It was found in this thesis that the p53 protein level increased following exposure to UVB and Se supplementation of cells did not prevent this accumulation. This observation does not dismiss the possibility, that Se may modulate the activity of the stabilised p53 protein and therefore decrease DNA binding and induction of apoptosis.

In the skin it would appear that the p53, Fas and TNF pathways are involved in the induction of apoptosis following exposure to UVB (Gniadecki *et al*, 1997). Therefore Se may modulate any one or more of these pathways. It was not possible to study the Fas pathway, this is unfortunate as the Fas pathway appears to be activated by ROS, formed following exposure of cells to UVB (Gorman *et al*, 1997). However it was possible to investigate the TNF pathway further, and it was discovered that Se pre-treatment decreased the UVB induction of TNF-α mRNA (Fig 6.12) and the protein for TNF-α by primary human keratinocytes (Fig 6.20). Therefore, there may be a decrease in the TNF-α response to UVB, which may be one mechanism by which Se decreases the levels of UVB-induced apoptosis (Schwartz, 1995).

Another possible mechanism by which Se may be decreasing cell death, apoptosis and tumour initiation, is by decreasing the level of UVB-induced DNA damage. Direct DNA damage in the form of CPDs and 6-4 photoproducts, are the most abundant form of DNA damage induced by UVB. DNA lesions such as these are linked to skin cancer formation and analysis of DNA from BCCs and SCCs has revealed that the majority of mutations occur at these sites. The significance of UVB-induced oxidative DNA damage in tumourogenesis is not fully known, but it does appear to contribute to the cells total burden of UVB-induced DNA damage (van der Scroeff *et al*, 1990; Nishigori *et al*, 1994). Selenium supplementation has been reported to decrease the formation of the oxidative DNA damage lesion 8-OHdg, in mouse keratinocytes following exposure to UVB (Stewart *et al*, 1996).

In my study the influence of Se on both direct and indirect forms of DNA damage were investigated. First, the impact of Se on the rate of excision repair of UVB-induced DNA damage was investigated. Selenium supplementation of primary keratinocytes was found not to alter the overall rate of excision repair (Fig 5.4). Pre-treatment with Se did not alter the formation of CPDs or their repair rate (Fig 5.6 and 5.7). However pre-treatment of primary keratinocytes with 50 nM sodium selenite or 200 nM selenomethionine, decreased the formation of 8-OHdg sites in irradiated primary human keratinocytes (Fig 5.12a and b). Therefore, Se supplementation may decrease the level of UVB-induced apoptosis, by decreasing the level of UVB-induced oxidative DNA damage. Both the hydroxyl radical and lipid peroxidation have been reported to mediated the formation of 8-OHdg sites in DNA (Peak and Peak, 1990; Park and Floyd, 1992). As previously mentioned in Chapter 3, Se can decrease the level of UVB-induced lipid peroxidation, subsequently this may then lead to a decrease in the formation of 8-OHdg sites in DNA.

Another significant effect of Se in studies on patients described in the literature is, the prevention of the inflammatory response following exposure to UVB (Burke *et al*, 1992a and b); this has also been reported to occur with mice (Overvad *et al*, 1985; Thorling *et al*, 1983). The effect of Se on the principal inflammatory cytokines in the skin was investigated. These include IL-6, IL-8, and IL-1α. Whilst Se decreased the UVB-induction mRNA for IL-6 (Fig 6.10a and b); and mRNA levels for IL-8 (Figs 6.11a and b); such effects were not repeated in the levels of cytokine proteins (Fig 6.18 and 6.19). The reason for the lack of modulation on the protein levels of these cytokines was unclear. Se did not alter the levels of IL-1α mRNA (Fig 6.13). Thus, despite my work it is still unclear how Se can modulate the inflammatory response, following exposure to UV. It is possible that Se may decrease prostaglandin synthesis, through the actions of selenoproteins, or decrease the levels of intracellular ROS which can act as mediators of inflammation.

Following initiation of skin neoplasms, the tumour which is highly immunogenic must evade the host immune response to progress and grow. Margaret Kripke and co workers were the first to report that UV-induced

tumours from mice transplanted to normal syngenic mice, which had been UV-irradiated, led to successful tumour take and growth (Fisher and Kripke, 1977; Kripke 1984). Since then, it has been reported that UV can cause both local and systemic immune suppression. Immune suppression allows tumours to progress, therefore the influence of Se on both local and systemic immune suppression was investigated. The main mediator of systemic immune suppression is IL-10 (Moore *et al*, 1993). IL-10 may also have a role in controlling the inflammatory response, which is induced following exposure to UVB. Irradiated murine keratinocytes secrete IL-10 protein (Rivas and Ullrich, 1992), however in human skin the IL-10 protein which is induced by exposure to UV is thought to be released by melanocytes or from infiltrating dermal macrophages (Teunissen *et al*, 1997; Mattei *et al*, 1994). Selenium supplementation of mouse keratinocytes decreased the UVB-induction of the protein for IL-10 (Fig 6.21). This suggests that a Se-mediated decrease in IL-10 protein, may be a mechanism by which the systemic immune suppression induced following UVB exposure could be abated, thus allowing a more effective immune defence to be made against tumour cells. The IL-10 protein also appears to be involved in local immune suppression, as it can inhibit antigen presentation within the skin and inhibits macrophage synthesis of inflammatory cytokines (Beissert *et al*, 1995; Niizeki and Streilein, 1997). Thus Se may also be able to modulate local immune suppression

One of the main inducers of local immune suppression in the skin is TNF-α. TNF-α is implicated in the migration of LCs from the epidermis, following exposure to UVB (Cumberbatch and Kimber, 1992; Yoshikawa, 1992; Cumberbatch and Kimber, 1995). Selenium supplementation of mouse keratinocytes decreased the UVB-induction of the mRNA for TNF-α. The UVB-induction of the human TNF-α protein was shown to be decreased following Se supplementation (Fig 6.20). The decrease in TNF-α protein levels would perhaps lead to a decrease in local immune suppression, due to the absence of the associated signal for LCs to migrate from the epidermis following exposure to UVB. Although it is not clear that there is a straight forward relationship between LC numbers in the epidermis and APC capacity, this is due to the presence of other dendritic cells in the skin (Duthie *et al*, 1999).

It was found that increased dietary Se intake in mice, decreased the UVB-induced loss of epidermal LCs (Fig 7.2). Another possible explanation for the retention of LCs in the skin is that LCs are susceptible to oxidative damage (Horio and Okamoto, 1987; Iwai *et al*, 1999), therefore Se via selenoprotein augmentation, may provide protection for LCs from oxidative damage. Finally, LCs have been shown to undergo apoptosis following exposure to UVB, and in Chapter 4, Se was shown to decrease the level of UVB-induced apoptosis in keratinocytes, therefore it is possible that Se could decrease the level of UVB-induced apoptosis in LCs.

Throughout this thesis, it has been proposed that Se exerts its actions via increased expression of antioxidant selenoproteins. Therefore, it was decided to investigate the level of selenoproteins found in the skin and to attempt to study the influence of dietary Se on these selenoproteins. The major antioxidant selenoproteins are the GPX and TR families. Glutathione peroxidase consists of a family of selenoproteins, the two most common ones in the skin are PHGPX and cGPX. Cytoplasmic glutathione peroxidase detoxifies hydrogen peroxide (Roetruck *et al*, 1973). Whereas, PHGPX can metabolise phospholipid hydroperoxides and lipid peroxides (Ursini *et al*, 1985; Maiorino *et al*, 1991). Thioredoxin reductase in conjunction with its substrate thioredoxin, can detoxify hydrogen peroxide (Bjornstedt *et al*, 1995a) and superoxide (Schallreuter and Wood, 1986). Thioredoxin reductase can also modulate other antioxidant systems in the skin. It has been reported to induce superoxide dismutase (Das *et al*, 1997) and can regenerate ascorbate (May *et al*, 1997). The regenerated ascorbate can then go on to regenerate α-tocopherol (Tamura *et al*, 1995). Finally TR can also act as an electron donor for GPX in the reduction of hydroperoxides (Bjornstedt *et al*, 1994).

Increasing Se levels in mice lead to increased activity of GPX in the livers (Fig 7.4), skin (Fig 7.5) and lymph nodes (Fig 7.6). These results demonstrate that, in mice, dietary Se reaches the skin and increases the levels of selenoproteins within it. The selenoprotein profiles of the various skin cell types were also investigated. Primary human keratinocytes, melanocytes and fibroblasts were shown to each express at least 10 major selenoproteins (Fig 8.1). All three cell types were confirmed to express PHGPX and TR by western immunoblotting. Exposure to UVB, did not alter the levels or

pattern of expression of any of the selenoproteins (Figure 8.5). Other groups have reported in the literature, that the activity of various selenoproteins increases in skin cells following Se supplementation. For example TR and GPX activity increase when 20 nM sodium selenite is added to the media of keratinocytes (Marcocci *et al*, 1997). The level of GPX and total Se have been shown to increase in patient's skin following Se supplementation (Burke *et al*, 1992a and b) and the same occurs in mouse skin (Pence *et al*, 1994).

The work presented in this thesis answers some of the questions relating to the role of Se in the skin. The data reported and discussed here, outlines the crucial role Se has in protecting the skin from a wide spectrum of UVB and UVA-induced damage. In a world of increasing exposure to UV radiation, due to a number of cultural and environmental factors, it is important that the role of Se in skin function is investigated further. Interesting areas for further research would be: to investigate the role of GPX and TR in protecting the skin from the harmful effects of UV, study further the role of Se in protection from immune suppression, to investigate the mechanism by which Se is involved in apoptosis, for example; is Se involved in the Fas pathway, can Se alter the activation of other downstream apoptotic genes e.g. Bax and Bcl-2 or does it alter the activation of the caspase proteins? Finally, a very important area of study would be, to look at the effect of Se *in vivo* using human trials and monitor if Se modulates the effects of UV on the skin.

From the work carried out in this thesis, it is not certain that Se supplementation would decrease the incidence of skin cancers. However, Se supplementation *in vitro* does decrease UVB-induced damage to skin cells. It would appear that sodium selenite is effective at lower concentrations, than selenomethionine, however selenite is also more toxic than selenomethionine. Therefore, for Se supplementation in humans selenomethionine may be preferred. Selenium supplementation has been shown to improve many other diseases and cancers including; prostate, colorectal and liver cancers, male infertility and cardiovascular disease. Therefore there may be a case for intervention to increase the Se intake in the UK up to at least the recommended level of 70 μg/day.

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Selenium: an essential element for immune function

Roderick C. McKenzie, Teresa S. Rafferty and Geoffrey J. Beckett

The importance of selenium for optimal immune function is now apparent. Here, Roddie McKenzie and colleagues describe how selenium is involved in the function of immune cells, and the various immune deficiencies and diseases that result from inadequate dietary intake.

elenium (Se) was discovered in 1817 by Jons Jacob Berzelius who named it after Selene, the Greek goddess of the moon. It toxic metaloid with a wide range of strial applications, including the pro-ion of semiconductors, photocopiers, less steel and antidandruff shampoos. 957 it became evident that Se was an ntial trace element that prevented tic necrosis in vitamin-deficient rats[1]. umans, clinical signs of toxicity (sel-s) appear when dietary intake exceeds g/day, an intake that can be exceeded untries where high concentrations of Se ound in the soil. A Se intake of up to g/day is regarded as safe[1].

he earliest evidence that Se is involved in une function came in 1959 with the ob-tion that dogs injected with ^{75}Se incor-ed the isotope into a leukocyte protein; rotein is now known to be cytoplasmic thione peroxidase (cGPX). In sheep and ns, Se is concentrated in tissues in-d in the immune response, such as n, liver and lymph nodes[2]. Later it was ciated that the trace element was essen-r normal growth and development and also prevent myopathies associated nutritional status in farm animals. In of China where there is marked Se de-cy, a severe endemic cardiomyopathy n as Keshan disease and a deforming arthritis known as Kashin–Beck disease und[1]. Both these diseases respond to Se ementation. Many studies suggest that uate intake of Se is required to ensure al immune function and to prevent ma-ncy. Various components of the immune n fail to function correctly if dietary Se icient[2] (Box 1).

e discovery in 1973 that Se was an in-part of the selenoenzyme cGPX pro-a mechanism by which Se could exert ological actions; this enzyme detoxifies ful organic hydroperoxides, as well as gen peroxide, which are produced g oxidative metabolism (reviewed in Refs 1–4). However, more-recent work suggests that cGPX is less important in preventing oxidative damage in the absence of stress and may simply provide a buffer to ensure a continued Se supply when dietary intake is limited. Other GPXs and selenoenzymes appear to be essential in preventing oxidative damage to the cell whereas some selenoproteins, such as the iodothyronine deiodinases, have roles that are unrelated to detoxification (reviewed in Refs 1, 3, 4).

Several studies have suggested that the incidence of malignancy and cardiovascular disease is inversely related to Se intake, but the precise means by which Se protects against these diseases is still unclear[1]. If Se does have marked effects on health and well-being, it is of concern that in many countries dietary Se intake is well below recommended levels[5].

Sources of Se

Ultimately, the soil is the source of Se; it enters the food chain through incorporation into vegetable protein as the amino acids selenocysteine and selenomethionine. Thus, if the animal and human population eat predominantly food produced locally, the Se status of the population will reflect soil levels. In many parts of Europe Se intake is low (30–40 μg/day[1,5]). The recommended dietary intake (RDI) for Se in the UK is 75 μg/day for adult males and 60 μg/day for adult females. In many parts of the USA, intake is above the RDI at 90 μg/day. In Finland, the low Se status of the livestock and human population, together with the high prevalence of skeletal muscle myopathy in animals, as well as heart disease and malignancy in humans, prompted a Se supplementation programme whereby inorganic Se was introduced into the food chain via fertilizers. This programme has increased Se intake in the Finnish population to ~125 μg/day.

Food contains organic forms of Se such as selenomethionine and selenocysteine but many experiments involving Se supplementation have used inorganic forms such as selenite. Absorption and use of organic and inorganic Se compounds differ. Organic forms appear to be re-used by the body more efficiently than inorganic forms, apparently because selenomethionine substitutes non-specifically for methionine residues in proteins but in such circumstances the Se has no bioactivity. For bioactivity and synthesis of specific selenoproteins, the trace element must be present as a selenide-like intermediate that incorporates into specific selenocysteine residues, usually at the active site of the selenoprotein[1,3,4]. Evidence suggests that selenide is more-readily formed from inorganic rather than organic Se. Furthermore, selenite but not selenomethionine reacts readily with glutathione in erythrocytes to form selenodiglutathione[6], a compound that has anticarcinogenic properties and induces apoptosis of human tumour cells[7].

Most Se present in tissues and blood is incorporated into selenoproteins, leaving little free Se. Many *in vitro* experiments have used selenocompounds at concentrations that are nonphysiological in that they reflect total rather than free circulating levels of Se. Circulating Se levels are low in many diseases, but this does not imply that Se is involved in the pathogenesis of the diseases. In many illnesses, trace elements such as zinc fall as part of the acute-phase response.

Selenoproteins and protection from oxidative damage

Selenoproteins are present in every cell type. At least 20–30 selenoproteins exist, but

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Box 1. Effects of Se on immune cell function

Se supplementation

In vivo

- Neutrophil migration and $O_2^{\cdot}$ activity (cow)
- High-affinity IL-2 receptor (mouse)
- T-cell proliferation and function following age-related decline (mouse)
- Natural killer cell activity (mouse, human)
- Cytotoxic T-cell activity (mouse)
- T-cell response to pokeweed mitogen (cow)
- Lymphokine-activated killer cell activity
- Enhanced delayed-type response due to better antigen presentation (mouse)
- Cell death following paraquat exposure (rat)
- UV-induced skin cancers and mortality (mouse)
- Erythema following UV exposure (human, mouse)
- Vaccine-induced immunity to malaria (mice)

In vitro

- HIV long-terminal-repeat activation and HIV replication in T cells (human)
- NF-κB activation (human)
- B-cell lipoxygenase activity (human)
- Antibody responses (primary and secondary) to virus (cow)
- Cell death following UV irradiation of skin cells (mouse, human)
- DNA damage and lipid peroxidation in UV-exposed skin cells (mouse, human)
- IL-6, IL-8 and TNF mRNA following UV treatment of skin cells (human)
- Cell death following paraquat exposure (human)
- Apoptosis in tumours (human, mouse)
- Apoptosis induced by UV in normal skin cells (human)
- Phytohaemagluttinin response in lymphocytes (human)
- Killing by macrophages (human)
- Target killing by cytotoxic T cells (human)

Se deficiency

- Platelet aggregation and leukotriene synthesis (atopic human)
- IgG and IgM titres (human)
- Antibody production by lymphocytes (mouse)
- Virulence of Coxsackievirus (mouse)
- Neutrophil chemotaxis (goat)
- Neutrophil and leukocyte activity (pig)
- Candidacidal activity by neutrophils (rat)
- $CD4^+$ T cells, ↓ $CD8^+$ T cells, ↓ $CD4^-/CD8^-$ thymocytes (mouse)

Abbreviations: HIV, human immunodeficiency virus; IL, interleukin; TNF, tumour necrosis factor; UV, ultraviolet radiation; ↑, increase; ↓, decrease.

about 12 have been either partially or pletely characterized (reviewed in Refs 4) and most appear to catalyse oxido- ction reactions[1,3,4]. It is widely held that ges in selenoprotein expression explain y of the biochemical and clinical mani- ions of selenium deficiency, although t effects of selenium compounds on cannot be excluded. Immunologically, bility of selenoproteins to protect the from oxidative stress is vitally impor- tant, since many host defence systems rely on the microbiocidal effects of macrophage- or neutrophil-generated free-radical species. Oxidative species are generated through general metabolism, during the metabolism of xenobiotics and during exposure to ultraviolet radiation (UV) in sunlight. Inflammation as a process to clear infection and damaged tissue also generates great oxidative stress. If antioxidant systems are not functioning correctly, host cells will be damaged.

Much is known about the functions of the family of GPXs. The membrane-bound phospholipid hydroperoxide GPX (PHGPX) detoxifies phospholipid hydroperoxides and, along with vitamin E, helps prevent oxidative damage to membranes. The PHGPX may be more important than the cGPX in protecting the cell from oxidative stress. Elimination of peroxides in the extracellular fluid is dealt with by the extracellular or plasma form of GPX. Peroxynitrites, products of superoxide and nitric oxide, are produced in skin cells during exposure to UV and cause single-strand DNA breaks. The synthetic Se compound ebselen functions as a GPX and prevents these breaks[8]. Ebselen also inhibits the proinflammatory enzymes nitric oxide synthase and protein kinase C (Ref. 9). In addition, the GPXs play a vital role in the synthesis of arachidonic acid metabolites. The lipoxygenase and cyclooxygenase pathways produce hydroperoxyeicosatetraenoic acids, which must be reduced for lipoxin, prostaglandin and leukotriene synthesis[2]. Eicosanoid synthesis is depressed in Se deficiency (see Ref. 2). Furthermore, accumulation of lipoperoxides impairs prostacyclin synthesis and promotes thromboxane accumulation, which can increase platelet aggregation in cardiovascular disease[5].

Other selenoproteins include the iodothyronine deiodinases (types I, II and III), which regulate the metabolism of thyroid hormones in all tissues. Clear functions are still being sought for other selenoproteins such as selenoprotein P and selenoprotein W. The former may be involved in Se transport; the latter is lost in Se-deficiency-induced myopathy[1,3,4].

The discovery that thioredoxin reductase (TDR) is a selenoenzyme is of great interest. One of its substrates is the 12 kDa thiol-protein thioredoxin[10] on which it acts as a protein disulphide reductase. TDR acting alone or in conjunction with thioredoxin can thus affect the redox regulation of a variety of key enzymes, transcription factors and receptors including ribonucleotide reductase, the glucocorticoid receptor, AP-1 and NF-κB. Thioredoxin is classified as a T-cell leukaemia survival factor because it stimulates expression of the α subunit of the interleukin 2 (IL-2) receptor[10]. In addition to

Box 2. Human diseases affected by Se or correlated with Se status

e deficiency
eshan disease (endemic cardiomyopathy)
topic asthma – low platelet glutathione peroxidase
ashin–Beck disease (endemic deforming arthritis)
ronary heart disease – correlates directly with low serum Se
IDS – low serum Se correlated with rapid disease progression
ontaneous abortion – correlated with low Se status
oriasis – severity and duration of disease correlated directly with decreased blood e levels
kin cancers – abnormally low serum Se in T-cell lymphoma and malignant elanoma
yxedematous cretinism – low serum Se
e supplementation
eduction in gastrointestinal, prostate and lung cancers (Se-replete population)
epressed neutrophil activity and increased monocyte chemoattractant protein in e elderly
rotection against hepatitis B-induced hepatoma
mproved sperm motility in subfertile men
ecrease in lipid peroxidation following UV exposure

r abbreviations, see Box 1.

cing thioredoxin, TDR can break down operoxide and lipid peroxides in the ence of NADPH more efficiently than can. Some anticarcinogenic effects of ppear to be mediated via TDR (Ref. 11).

cts of Se on immune function

eficiency depresses the effectiveness of une cells generally, with diverse spe- effects listed in Box 1. Supplementation Se appears to boost cellular immunity hree mechanisms. First, it upregulates xpression of the T-cell high-affinity IL- eptor[12] and provides a vehicle for en- ed T-cell responses. Since the T cell is a component in providing B-cell help for ody synthesis, this may explain the ulatory effects of Se on antibody pro- on (Box 1). In fact, age-related de- es in cellular immunity can be partially sed by Se supplementation increasing nsiveness to IL-2 (Ref. 13). Second, it ents oxidative-stress-induced damage mune cells. Third, it alters platelet ag- tion by decreasing the ratio of throm- ne to leukotriene production. Human ses associated with Se deficiency are in Box 2 and below.

Selenium and viral diseases

In mice, the coxsackievirus mutates to a virulent cardiotoxic form when it is passaged through Se-deficient hosts[14], possibly because the host immune system is weakened. The fact that human Keshan disease is seen in Se-deficient areas of China led to the hypothesis that it may be caused by a coxsackie B virus that mutates to a cardiotoxic form in Se-deficient hosts. This disease responds to Se-dietary supplementation. Similarly, in China, the incidence of hepatitis-B-induced hepatoma fell after Se supplementation[15]. In AIDS patients, Se status is predictive of survival times[16]. There is also the intriguing observation that virulent strains of influenza virus evolve in Se-deficient areas of China and the suggestion that human immunodeficiency virus (HIV) may have crossed the species barrier into man in Se-deficient areas of Africa. Selenite also seems to protect mouse cells in culture from the transforming effects of murine mammary tumour virus. Interestingly several viruses (molluscum contagiosum, HIV-1, coxsackie B and Ebola) contain sequences with homology to GPX (Ref. 17); perhaps these viral GPXs might be involved in protecting the virus from host-cell-derived peroxides.

Se and protection from UV-induced damage and cancer

UV is the most ubiquitous environmental carcinogen. Skin exposure leads to DNA, lipid and protein damage by direct and free-radical-mediated effects. In mice, dietary Se supplementation reduces incidence of and mortality from UV-induced non-melanoma skin cancers. Se supplementation prevents DNA adduct accumulation, cell death, lipid peroxidation and inflammatory cytokine induction in skin cells in culture following UV irradiation[18]. Transfection with a viral GPX also improves keratinocyte survival following UV exposure[19].

An exciting finding is that doses of 200 μg/day selenomethionine reduce the overall incidence of prostate, lung and gastrointestinal cancers by 39% (Ref. 20). These effects may result from a number of mechanisms including preventing oxidative damage to DNA, maintaining effective immune defences and inhibiting tumour proliferation.

Conclusion

In conclusion, adequate Se is essential for immune function and can protect the immune system from oxidative damage. Dietary Se supplements hold promise as a means of treating inflammatory conditions and rejuvenating the ageing immune system, and in protection from carcinogenesis.

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Roderick McKenzie *(Roddie.McKenzie@ed.ac.uk) and* ***Teresa Rafferty*** *are at the Dept of Dermatology and* ***Geoffrey Beckett*** *is at the Dept of Clinical Biochemistry, University of Edinburgh, Scotland, UK EH3 9YW.*

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rential expression of selenoproteins by human skin cells and protection elenium from UVB-radiation-induced cell death

. RAFFERTY[1]*, Roderick C. McKENZIE*, John A. A. HUNTER*, A. Forbes HOWIE†, John R. ARTHUR‡, Fergus NICOL‡ ff J. BECKETT†

t of Dermatology, University of Edinburgh, Lauriston Place, Edinburgh EH3 9YW, Scotland, U.K., †Department of Clinical Biochemistry, University of Lauriston Place, Edinburgh EH3 9YW, Scotland, U.K., and ‡Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, Scotland, U.K.

ration of reactive oxygen species has been implicated as he mechanism responsible for UVB-radiation-induced nage. In mice, evidence suggests that increased dietary intake may protect skin from many of the harmful UVB radiation. We sought to determine the seleno-rofile of cultured human skin cells and whether selenium ntation could protect keratinocytes and melanocytes lethal effects of UVB radiation. Labelling experiments Se]selenite showed qualitative and quantitative differ-selenoprotein expression by human fibroblasts, keratin-d melanocytes. This was most noticeable for thioredoxin (60 kDa) and phospholipid glutathione peroxidase ; these proteins were identified by Western blotting. hese differences, we found that a 24 h preincubation with sodium selenite or selenomethionine protected both cultured human keratinocytes and melanocytes from UVB-induced cell death. With primary keratinocytes, the greatest reduction in cell death was found with 10 nM sodium selenite (79% cell death reduced to 21.7%; $P < 0.01$) and with 50 nM selenomethionine (79% cell death reduced to 13.2%; $P < 0.01$). Protection could be obtained with concentrations as low as 1 nM with sodium selenite and 10 nM with selenomethionine. When selenium was added after UVB radiation, little protection could be achieved, with cell death only being reduced from 88.5% to about 50% with both compounds. In all of the experiments sodium selenite was more potent than selenomethionine at providing protection from UVB radiation.

CTION

alence of non-melanoma skin cancer is increasing, and ve ultraviolet B radiation (UVB) (280–320 nm) dose is accepted as being one of the most important aetiological UVB is a complete carcinogen; it can both initiate and carcinogenesis. Two of its major effects are DNA nd local and systemic immune suppression [1,2]. The n of free radicals [3] and cytokines [4] has been d as being partly responsible for these effects. The skin many enzyme systems to prevent free-radical-induced including catalase, superoxide dismutase and selenium aining glutathione peroxidases (GPXs). Catalase and le dismutase activity are decreased following UVB while GPX appears to be resistant to these effects [5]. s much evidence to suggest that Se has an important otecting skin from the harmful effects of UVB. In mice, ementation can substantially decrease the amount of age, tumour formation and overall mortality following osure [6,7]. In humans, subnormal Se status is associated o a 4-fold increased risk of developing skin cancer [8], al Se application, as selenomethionine, has been shown t subjects from acute skin damage following UVB [9].

actions of Se were once thought to be exerted through GPX, but it is now recognized that Se exerts many of through the expression of a number of selenoproteins, Se is covalently linked within the protein as seleno-10]. At least 30 selenoproteins have been identified by GE of ^{75}Se-labelled tissue. However, only approximately 14 have been characterized, including cytosolic GPX, phospholipid hydroperoxide GPX (PHGPX), extracellular GPX, selenoprotein-P, SP56, protein disulphide isomerase, thioredoxin reductase (TR) and the iodothyronine deiodinases [11,12]. The effects of UVB on the expression of these selenoproteins, or indeed their precise roles in influencing the cells' response to UVB, are not known. The family of GPXs detoxifies a wide range of lipid peroxides [13], and these enzymes have been implicated in protecting cells from UVB-induced free-radical damage. TR is an FAD-containing enzyme found in all organisms which, in conjunction with its substrate thioredoxin, forms a redox system which has multiple functions, including detoxification reactions [14–16].

Labelling experiments with ^{75}Se have shown clear tissue differences in the pattern of selenoprotein expression [17], but studies have not been performed on human skin. Such studies may indicate which selenoproteins have a role in protecting skin from the harmful effects of UVB.

Both inorganic and organic forms of Se are commonly used as Se supplements in experiments, although dietary Se is found predominantly as the organic forms selenomethionine and selenocysteine [18]. In humans, whole-body turnover studies indicate that selenomethionine and selenocysteine are re-utilized more efficiently than inorganic forms such as sodium selenite [19]. This occurs because selenomethionine can be substituted non-specifically into proteins at methionine residues. However, inorganic forms of Se may provide a more bioactive form of the trace element than selenomethionine and, in cultured thyrocytes, sodium selenite is retained and utilized by an energy-dependent process [20]. Studies have not been performed to compare the

tions used: UVB, ultraviolet B radiation; GPX, glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase; TR, reductase; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earle's balanced salt solution.

om correspondence should be addressed (e-mail tr@srv1.med.ed.ac.uk).

tency of sodium selenite and selenomethionine in protecting tured human skin cells from UVB.

Here, for the first time, we define the profiles of selenoproteins pressed by cultured human keratinocytes, melanocytes and roblasts. We also show that sodium selenite and seleno-thionine supplementation protects human melanocytes and atinocytes *in vitro* from UVB-induced cell death.

TERIALS AND METHODS

terials

lbecco's modified Eagle's medium (DMEM), penicillin/ptomycin, glutamine, dispase, trypsin, sodium pyruvate, atinocyte serum-free medium, Earle's balanced salt solution BSS) and the culture vessels were purchased from Gibco BRL, sley, Renfrewshire, Scotland, U.K. [^{75}Se]Selenite (33 MBq/) was purchased from the University of Missouri, Columbia,), U.S.A. All other chemicals and reagents and the melanocyte ure media were purchased from Sigma-Aldrich, Poole, rset, U.K. Antibodies to rat liver TR and rat PHGPX were ed in rabbits using proteins purified as described previously 21].

ue culture

tures of the spontaneously transformed keratinocyte line CaT [22] (a gift from Professor N. Fusenig, Differenzierung Carcinogenese, Deutsches Krebsforschungszentrum, Heidel-g, Germany), were grown in DMEM supplemented with 5%) foetal calf serum, 100 units/ml penicillin and 100 μg/ml ptomycin in a humidified 5% CO_2 atmosphere at 37 °C. nary fibroblasts were cultured from forearm biopsies [23], vn out from underneath coverslips and cultured until con-nt. These were grown in DMEM as above, but with 10% al calf serum.

rimary human keratinocytes and melanocytes were cultured n neonatal foreskins as described previously [24]. Briefly, the skin samples were incubated overnight in 0.1% dispase/EM. The epidermis was removed and dispersed in trypsin g/l) and EDTA (6.8 μM) for 15 min. Cells were then plated on fibronectin-coated plates and grown in either keratinocyte nelanocyte medium. After two passages, the cultures were posed predominantly (> 95%) of either keratinocytes or nocytes respectively. The cells were used at passages 3 or 4. Se content of each culture medium was determined by acid stion followed by fluorimetric analysis [25] and the following lts found: melanocyte medium, 28 nM; keratinocyte me-n, 8.9 nM. No Se was detected in the DMEM with 5 or 10% al calf serum added.

rmination of selenoprotein profiles

iles of selenoproteins expressed by the various primary skin were determined by radiolabelling with [^{75}Se]selenite as ribed previously [26]. Briefly, confluent cultures of cells in m^2 flasks were labelled with 0.02 MBq/ml [^{75}Se]selenite by bation for 72 h. Previous studies have shown that ^{75}Se ke and incorporation into selenoproteins by cells reaches a au at approx. 24 h, and therefore cells were labelled for 72 h nsure that a steady state had been reached. Medium was nted and cells were washed twice with EBSS before being ested into EBSS by scraping, followed by centrifugation at *g* for 10 min. Cells were resuspended in 60 mM Tris buffer, .8, containing 1 mM dithiothreitol and 1 mM EDTA, and by sonication. Equal amounts of protein (25 μg) were loaded on to SDS/PAGE (12% gels) and proteins were separated by electrophoresis. The gels were dried and the labelled selenoproteins were quantified using a molecular PhosphorImager (model GS-525; Bio-Rad, Hemel Hempstead, Herts., U.K.) and visualized by autoradiography using Kodak X-OMAT XAR-5 film. Selenoprotein profiles were also determined in primary melanocytes, keratinocytes and fibroblasts which had either (1) been prelabelled with ^{75}Se for 24 h prior to being exposed to 200 J/m^2 UVB and then harvested 24 h post-exposure, or (2) been exposed to 200 J/m^2 UVB, then labelled with ^{75}Se and harvested 24 h later.

Western blot analysis was used to identify TR and PHGPX. The proteins were separated by SDS/PAGE and were transferred to Immobilon P membranes. The blot was probed with affinity-purified polyclonal antibodies to rat liver TR and developed using chemiluminescence or coupled alkaline phosphatase reagents [27]. The blots were also probed with purified antibodies to rat PHGPX.

Protein determination

The protein content of the sonicated samples was determined using the Bradford dye-binding method [28] adapted for use on a Cobras Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, U.K.).

UV irradiation

To determine the lethal UVB dose for the various cell types, cells were grown to 70% confluence; the medium was then removed and set aside, and the monolayer was covered with 1 ml of PBS. Then the cells were irradiated with broadband UVB from a bank of two TL-20W/12 lamps (Philips, Croydon, U.K.) with an output range of 270–350 nm (peak at 308 nm). The irradiance was 80 mW/cm^2 at a distance of 30 cm [24]. To determine the UVB dose that killed 80% of the cells, doses of between 480 and 1200 J/m^2 were given. The original medium was replaced, and cell viability was determined by cell counts on a haemocytometer using Trypan Blue (0.4%, w/v) exclusion, 48 h after irradiation.

Effects of selenium supplementation on cell viability and growth

Cells grown in six-well dishes to 70% confluence were treated with sodium selenite or selenomethionine (1 nM–10 μM) and incubated for 72 h. The toxicity of these compounds was determined by assessing cell viability using Trypan Blue exclusion. Growth stimulation was assessed by counting cells using a Coulter counter (model DN; Coulter Electronics Ltd., Harpenden, U.K.).

Protective effect of selenium supplementation on cells treated with UVB

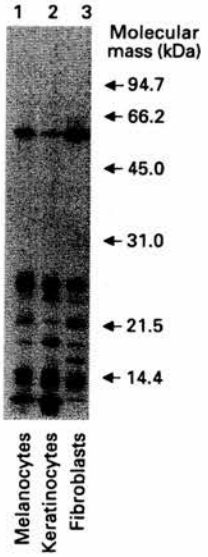
Cells cultured in six-well dishes until 70% confluent were treated with sodium selenite or selenomethionine (1 nM–1 μM), and incubated for 24 h. The medium was removed and set aside, and 1 ml of PBS was added to each well immediately prior to the cells being exposed to UVB. Typically the cells were exposed to UVB for 1.5–2 min (720–960 J/m^2; the dose given depended on the cell type used), which resulted in approx. 80% cell death 48 h post-irradiation, in cultures where no sodium selenite or selenomethionine had been added. After UVB exposure, PBS was removed and the original culture medium was replaced. Viability was determined by Trypan Blue exclusion 48 h post-UVB exposure.

econd series of experiments, cells were treated with selenite or selenomethionine immediately after UVB (without the 24 h pretreatment with Se). The rest of the nt was performed as above.

periments were carried out twice (with similar results), plicate samples for each data point. ANOVA and the test Bonferroni were used to analyse the data; values for were deemed to be significant. To compare the dif- between sodium selenite and selenomethionine, ANOVA *ost hoc* test Scheffe were used; again values for $P < 0.05$ med significant.

tein profiles in cultured melanocytes, fibroblasts and ytes

keratinocytes, melanocytes and fibroblasts were found learly different selenoprotein profiles (Figures 1 and 2). type showed up to 10 major selenoproteins (molecular ging from approx. 10 kDa to 60 kDa), but many minor teins were also expressed. Of particular note was the xpression between cell types of the 21 kDa and 60 kDa teins. Western blotting identified the 21 kDa and elenoproteins as being PHGPX and TR respectively ot shown). The 60 kDa ^{75}Se-labelled bands in each cell sponded exactly in mobility and relative intensity to the vealed by Western blotting. The Western blot was not nough to detect PHGPX directly; however, a standard PHGPX had exactly the same mobility as the 21 kDa led band. The greatest expression of TR and PHGPX in fibroblasts, with keratinocytes expressing relatively hese selenoproteins. PHGPX and TR expression was melanocytes than keratinocytes, but not as great as in s. At 24 h following exposure to UVB, no change in the n of any of the selenoproteins could be observed (results n).



Selenoprotein expression by primary human skin cells

ocytes; lane 2, keratinocytes; lane 3, fibroblasts. Cells were labelled with (0.02 MBq) for 72 h, and the proteins were separated by SDS/PAGE and utoradiography.

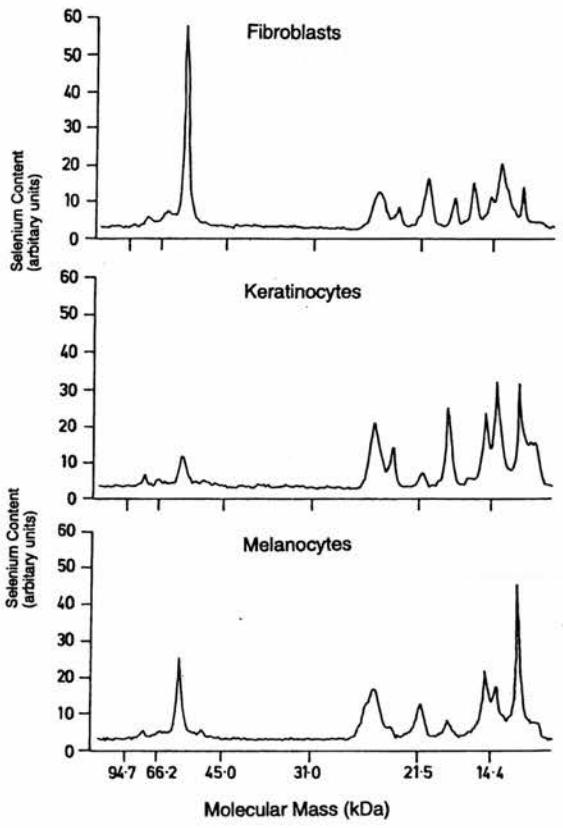


Figure 2 Selenoprotein profile analysis of primary human skin cells

A PhosphorImager was used to quantify the radioactivity of the selenoproteins expressed by the primary skin cells. The protein gels were scanned overnight on an imager and the profiles of selenium-containing proteins were generated. The molecular masses are shown on the bottom profile.

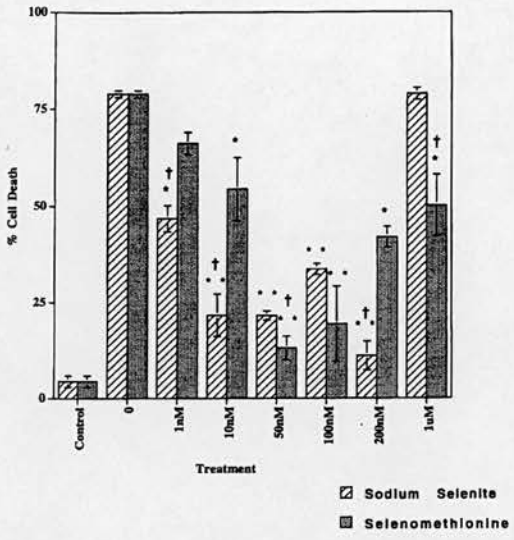
Table 1 Effects of sodium selenite and selenomethionine on the viability of primary human keratinocytes

Primary human keratinocytes were incubated with sodium selenite or selenomethionine for 72 h, and the viable cells were counted using a haemocytometer and Trypan Blue exclusion. Control cells had no Se added to them. At least 100 cells were counted. Results are means ± S.E.M. ($n = 3$). Significant differences from control: $^{*}P < 0.05$, $^{**}P < 0.01$.

	Cell death (%)	
Dose (μM)	Sodium selenite	Selenomethionine
Control	1.8 ± 1.8	1.8 ± 1.8
0.1	1.3 ± 1.8	1.8 ± 1.8
0.2	6.5 ± 1.2	1.2 ± 0.8
1	24.5 ± 1.5*	1.5 ± 0.3
10	56.0 ± 5.4**	5.4 ± 2.1

Effect of Se supplementation on cell growth and cell viability

In cultured primary keratinocytes, sodium selenite or selenomethionine had no apparent growth-stimulating activity (results not shown), and sodium selenite was only significantly toxic at concentrations of 1 μM or above (Table 1). Selenomethionine was not found to be toxic at concentrations up to 10 μM (Table 1).



re 3 Effect of sodium selenite or selenomethionine pretreatment on viability of primary human keratinocytes after exposure to UVB

ry keratinocytes were pretreated with sodium selenite or selenomethionine for 24 h; then edium was replaced with PBS and the cells were exposed to a dose of UVB (960 J/m^2). riginal medium was returned and the cells were incubated for 48 h before cell counts were med using a haemocytometer and Trypan Blue exclusion. Control cells received no UVB supplement. At least 100 cells were counted. Results are given as percentage cell death s ± S.E.M., $n = 3$). Significant difference from cells not treated with Se, but exposed to $^*P < 0.05$; $^{**}P < 0.01$. Significant differences between the sodium selenite and omethionine results are indicated by $\dagger P < 0.05$.

ilar results were found in primary melanocytes and HaCaT (not shown).

ct of selenium in decreasing UVB-induced cell death

dose of UVB used (960 J/m^2) produced 80% cell death in nary keratinocytes 48 h after exposure. The addition of um selenite or selenomethionine, prior to exposure to UVB, vented a significant amount of cell death, but the protective ct was significantly different between the two compounds ure 3). A protective effect with sodium selenite was seen with s from 1 to 200 nM, with an optimal protective dose of M. Selenomethionine was less potent than sodium selenite in ecting cells from UVB-induced damage, with a higher centration of selenomethionine than sodium selenite being ired to give significant protection from UVB-induced dam- No significant protective effect of selenomethionine was rved below 10 nM. Maximal protection using seleno- nionine was found at 50 nM, with concentrations in the e 10 nM–1 μM offering significant protection. As with um selenite, the protection afforded by selenomethionine ed to decrease at 1 μM.

elanocytes were more sensitive to UVB than were keratin- es, and a UVB dose of 720 J/m^2 was sufficient to kill 80% ne cells. With melanocytes, the effects of Se were similar to e seen with keratinocytes, with maximal protection being eved for sodium selenite and selenomethionine at concen- ons of 10 nM and 100 nM respectively, with little or no ection found at 1 μM (Table 2). In the keratinocyte cell line aT, a dose of 960 J/m^2 UVB was required to kill 80% of the and maximal protection with Se was found at 10 nM um selenite and 100 nM selenomethionine (Table 2).

experiments in which sodium selenite or selenomethionine

Table 2 Effects of selenomethionine and sodium selenite pretreatment on the viability of primary human melanocytes and the human keratinocyte cell line HaCaT after exposure to UVB

Primary melanocytes and HaCaT cells were pretreated with sodium selenite or selenomethionine for 24 h prior to the medium being replaced with PBS and the cells being exposed to a dose of UVB (melanocytes, 720 J/m^2; HaCaT cells, 960 J/m^2). The original medium was returned and the cells incubated for 48 h before cell counts were performed using a haemocytometer and Trypan Blue exclusion. Control cells received no UVB or Se supplement. At least 100 cells were counted. Results are the means ± S.E.M. ($n = 3$). Significant differences from cells not treated with Se, but exposed to UVB: $^*P < 0.05$; $^{**}P < 0.01$. Significant differences between the sodium selenite and selenomethionine results are indicated by: $\dagger P < 0.05$.

	Cell death (%)			
	Sodium selenite		Selenomethionine	
Dose (nM)	Melanocytes	HaCaT cells	Melanocytes	HaCaT cells
Control	7.7 ± 1.6	4.3 ± 4.3	7.7 ± 1.6	4.3 ± 4.3
0	82.6 ± 1.4	86.8 ± 1.1	82.6 ± 1.4	86.8 ± 1.1
1	57.7 ± 6.4*†	57.6 ± 2.9*†	79.3 ± 1.7	79.4 ± 3.8
10	10.3 ± 0.9**†	19.5 ± 1.4**†	72.4 ± 2.3*	64.3 ± 2.2*
50	22.5 ± 1.5**†	35.4 ± 1.9**	26.9 ± 1.5**	23.8 ± 4.5**
100	29.2 ± 1.1**	45.2 ± 2.8*	14.3 ± 4.9**	11.6 ± 3.9**†
200	34.4 ± 3.5**	60.4 ± 2.2*	30.3 ± 3.4**	39.5 ± 3.1**†
1000	75.4 ± 3.4	86.7 ± 2.3	65.6 ± 3.3*	75.2 ± 3.4

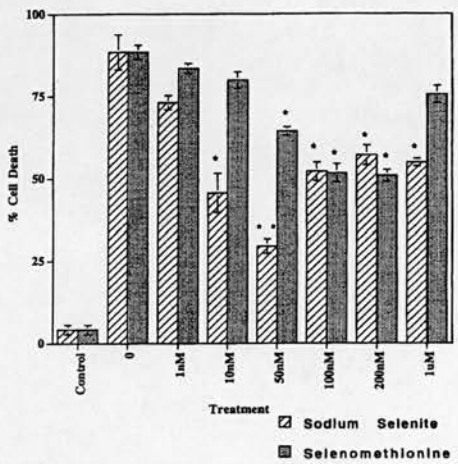


Figure 4 Effects of sodium selenite or selenomethionine added after the exposure of primary human keratinocytes to UVB

The medium on primary keratinocytes was replaced with PBS prior to the cells being exposed to a dose of UVB (960 J/m^2). Then the original medium was returned, with either sodium selenite or selenomethionine added, and the cells were incubated for 48 h before cell counts were performed using a haemocytometer and Trypan Blue exclusion. Control cells received no UVB or Se supplement. Results are given as percentage cell death (means ± S.E.M., $n = 3$). Significant differences from cells not treated with Se, but exposed to UVB: $^*P < 0.05$, $^{**}P < 0.01$.

was added to primary keratinocytes immediately after UVB exposure, protection was still achieved, but the protection was modest and required higher doses of Se compared with protection achieved by adding sodium selenite or selenomethionine to cells 24 h prior to UVB exposure (Figure 4). Maximal protection was achieved at 50 nM sodium selenite and 200 nM selenomethionine. The level of protection was much less (2–3-fold decrease in cell death) for the cells treated with Se post-UVB compared with that

ells pretreated with Se for 24 h (5–6-fold decrease in n) (Figures 3 and 4).

ION

lts presented here show that preincubation of primary of keratinocytes, melanocytes or the HaCaT cell line or 24 h can provide all of these cell types with significant n from UVB-induced cell death (Figure 3; Table 2). The form of Se added to cells was an important factor in ing the minimal and optimal doses of Se required to protection from UVB. Selenite was more potent than thionine at conferring protection, illustrated by the hat, in melanocytes, keratinocytes and HaCaT cells, t protection was achieved with a dose of 1 nM whereas no significant protection was achieved with thionine below concentrations of 10 nM. Concen- of Se that conferred maximal protection to cells also ith the chemical form of Se. With selenite, maximal n was achieved at a concentration of 10 nM in all cell ereas, with selenomethionine, maximal protection was at concentrations of 50 nM, 100 nM and 100 nM for ytes, HaCaT cells and melanocytes respectively (Figure 2). The observation that a similar degree of protection by Se addition was found with the HaCaT keratinocyte nd primary keratinocytes suggests that the phenomenon al one among skin cell types.

d not investigate the effects of Se on fibroblasts, as cies in the skin arise from keratinocytes and melanocytes wever, Richard et al. [29] have shown that fibroblasts otected from broadband UVB by selenium.

dely held that Se exerts many of its biochemical actions he expression of specific selenoproteins in which Se is s specific selenocysteine residues encoded by a TGA sertion of Se at these TGA-directed sites requires Se to ted in a chemically active form similar to selenide, and idence indicates that selenite is a more potent precursor e than is selenomethionine [30]. Furthermore, although dy turnover studies in humans have shown that seleno- ne is re-utilized by cells more efficiently than inorganic is appears to arise because selenomethionine can be d into proteins non-specifically for methionine. When methionine residues, selenomethionine appears to have vity [31]. Further evidence for the increased bioactivity e over selenomethionine comes from the observation ite, but not selenomethionine, can be converted within o selenodiglutathione, a form of Se which appears able e apoptosis [32].

gh selenite was more potent than selenomethionine at protection from UVB damage, our data clearly hat selenite was much more toxic to cells than was hionine. Thus 1 μM selenite killed approx. 25% of at a dose of 10 μM more than 50% of the cells were ; this contrasts with selenomethionine, which showed y at any of the doses used in the study (Table 1). While ay itself be directly toxic to cells, a study has shown etabolite selenodiglutathione is also a potent cytotoxic . Although both Se compounds conferred dose-de- rotection from UVB damage up to approx. 100 nM, appeared to be diminished in all cell types when the was further increased to 1 μM. This loss of protection asing concentration of selenite is most likely to be due ct toxic effect of selenite and its metabolite seleno- one when present at high concentrations. For seleno- e, the mechanism behind the loss of protection at higher doses is unknown, since selenomethionine was not toxic at any of the concentrations used (Table 1). It is possible that UVB exposure might modify selenomethionine to produce a toxic metabolite, but we could find no evidence of increased toxicity in a solution of selenomethionine irradiated with UVB (results not shown).

We have shown that preincubation with Se can prevent UVB-induced damage; the mechanism by which this occurs is not clear. Se did not exert a protective effect through altering cell growth patterns at the doses that were protective. If Se was added immediately after UVB exposure, some protection was achieved, but this effect was small compared with the protection achieved if the cells were preincubated with the trace element (Figure 4). These data suggest that Se may be acting through incorporation into selenoproteins rather than by a direct anti-oxidant chemical action.

The family of GPXs are clearly potential candidates for selenoproteins that may mediate the protective effects of Se, since these enzymes are capable of detoxifying hydrogen peroxide, lipid hydroperoxides and phospholipid hydroperoxides which are produced during UVB exposure. TR is a selenoenzyme which has multiple functions and can act through its ability to reduce oxidized thioredoxin [14] or its ability to directly reduce hydrogen peroxide and lipid hydroperoxides in the presence of NADPH [33]. Thus TR may play an important role in protecting cells from peroxidative damage. However, labelling of tissues with [^{75}Se]selenite has revealed more than 30 selenoproteins, and the pattern of selenoprotein expression is quite different between tissues. It is thought that some of these selenoproteins may also be involved in detoxification reactions and in modifying cell growth or cell death [30].

The expression of the 21 and 60 kDa selenoproteins showed the most diversity between cell types. These selenoproteins were identified by Western blotting to be PHGPX and TR. We found that keratinocytes expressed much lower levels of TR and PHGPX than melanocytes, and yet keratinocytes were found to be more resistant to UVB-induced cell death than melanocytes; typically, a UVB dose of 720 J/m^2 was required to produce 80% cell death in melanocytes, while a dose of 960 J/m^2 was required to produce 80% cell death in keratinocytes. Furthermore, despite these differences in selenoprotein expression between the two cell types, keratinocytes and melanocytes showed a similar dose requirement for selenium to obtain maximal protection. While it could be argued that these observations imply that the selenoproteins TR and PHGPX are not involved in protection, this is not necessarily the case. The susceptibility of a cell to UVB-induced damage will depend on the total expression of a wide range of antioxidant enzymes and DNA repair mechanisms, and not necessarily on differences in the expression of a single specific selenoprotein. Thus conclusions regarding the protective role of a specific selenoprotein made on the basis of comparison of the susceptibility of different cell types to UVB-induced cell death may be misleading; experiments that relate to the susceptibility of a cell to UVB-damage after modifying the expression of a single selenoprotein within a single cell type would provide more conclusive data.

We have shown that Se can protect both keratinocytes and melanocytes from UVB-induced damage, and such protection can be achieved using very low concentrations of Se, particularly when presented as sodium selenite. Intake of Se in the U.K. has fallen considerably over the past two decades; at present, the average intake is only about 40% of the recommended daily allowance (75 μg/day) [34]. It is possible that a contributing factor to the increase in the incidence of skin cancer of all types seen in the U.K. [35] may, in part, be due to this decline in Se

us. Our data from isolated cells would suggest that, as in the use [6], increasing the Se intake of humans could afford tection from the harmful effects of UVB radiation. Furmore, our results suggest that, if supplements of Se were to given, inorganic forms such as selenite may be more potent at viding this protection than organic forms such as selenohionine.

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Expression Of Selenoproteins In Human Skin Cells And Protection From Ultraviolet B Radiation-Induced Cell Death By Selenium Supplementation

Teresa S. Rafferty, Roderick C. McKenzie, John A.A. Hunter, A. Forbes Howie*, John R. Arthur, Fergus Nicol ** & Geoff J. Beckett***

Departments of Dermatology & *Clinical Biochemistry, University of Edinburgh, Edinburgh EH3 9YW and **Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB.

INTRODUCTION

The prevalence of non-melanoma skin cancer is increasing and cumulative ultraviolet B radiation (UVB) (280-320nm) dose is accepted to be one of the most important factors in the aetiology of the disease. The generation of free radicals [1] and cytokines [2] have been implicated as being partly responsible for these effects. Selenium (Se) has an important role in protecting skin from the harmful effects of UVB. In mice Se supplementation can substantially decrease the amount of skin damage, tumour formation and overall mortality following UVB exposure [3]. In humans low serum Se levels are associated with an increased risk of developing skin cancer [4] and topical Se application, as selenomethionine protects subjects from acute skin damage following UVB exposure [5].

Selenium is thought to act predominantly through the action of selenoproteins and at least 30 selenoproteins have been identified by SDS-polyacrylamide electrophoresis of [^{75}Se]-labelled tissue. However, approximately only 14 have been characterised, including cytoplasmic glutathione peroxidase (cyGPX), phospholipid hydroperoxide GPX (PHGPX), extracellular GPX, selenoprotein-P, AP56, thioredoxin reductase (TR) and the iodothyronine deiodinases [6,7].

Here we define the profiles of selenoproteins expressed by cultured human keratinocytes, fibroblasts and melanocytes. We also show that sodium selenite and selenomethionine supplementation protects human melanocytes and keratinocytes *in vitro* from UVB-induced cell death.

METHODS

The protective effect of Se on cells was determined as previously described [8]. Briefly cells were treated with sodium selenite or selenomethionine (1nM - 1μM) for 24 hours. The media was removed and set aside and phosphate buffered saline (PBS) added to each well immediately prior to the cells being exposed to broadband UVB. Typically the keratinocytes were exposed to 960J/m^2 of UVB and the melanocytes to

720J/m^2, which resulted in approximately 80% cell death in cultures where no sodium selenite or selenomethionine had been added. After UVB exposure PBS was removed and the original culture media was replaced. Viability was determined by trypan blue exclusion 48 hours post UVB exposure. In a second series of experiments, cells were treated with sodium selenite or selenomethionine immediately after UVB exposure (without the 24 hour pre-treatment with Se). The rest of the experiment was performed as above.

Profiles of selenoproteins expressed by the various primary skin cells were determined by radiolabelling with [^{75}Se]-selenite as described previously [9].

All experiments were carried out twice (with similar results), using triplicate samples for each data point. ANOVA and the posthoc test Bonferroni were used to analyse the data, values for $p < 0.05$ were deemed to be significant.

RESULTS AND DISCUSSION

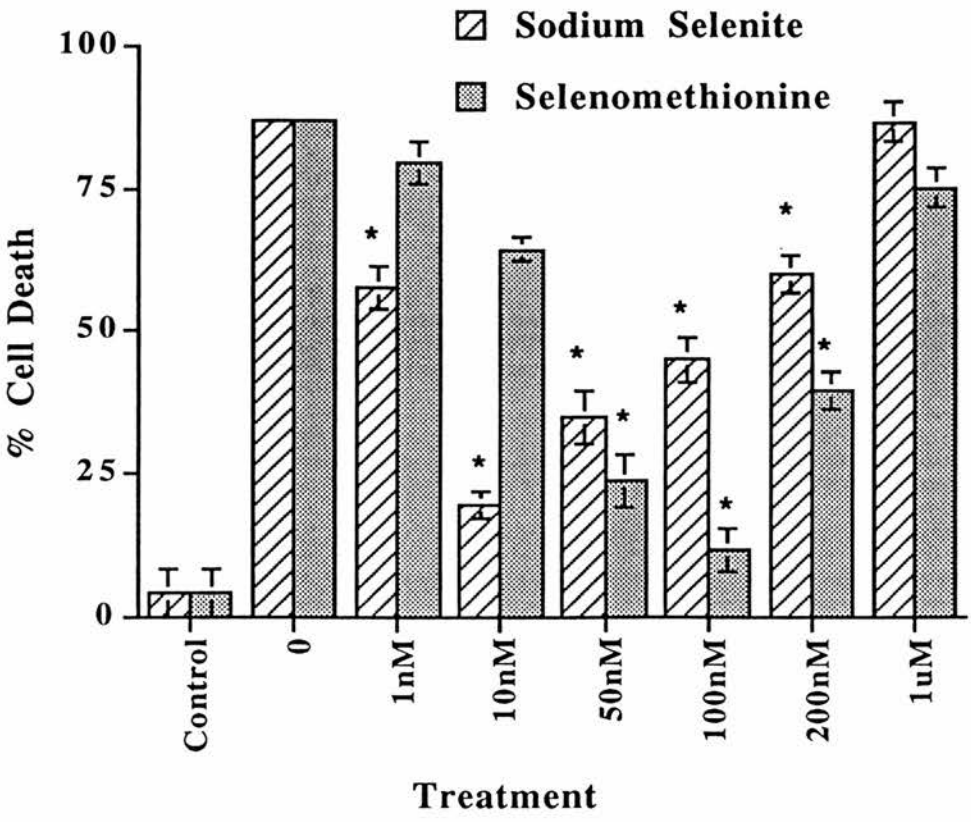

FIGURE 1 : The effect of Se pre-treatment on the viability of primary human keratinocytes after exposure to UVB.

Cells were pre-treated with sodium selenite or selenomethionine for 24 hours prior to being exposed to UVB (960J/m^2). Cell counts were performed using a haemocytometer and trypan blue exclusion 48 hours after UVB exposure. Control cells received no UVB or Se supplement. Results are the means of the percentage cell death ± S.E.M, (n = 3). Significant difference from cells not treated with Se, but exposed to UVB (= $p < 0.05$).*

The dose of UVB used (960J/m^2) produced 80% cell death in primary keratinocytes 48 hours after exposure, and pre-incubation of keratinocytes or melanocytes with Se for 24 hours provided both cell types with significant protection from UVB-induced cell death (Fig 1: data for melanocytes is not shown). Selenite was more potent than selenomethionine at conferring protection as illustrated by the finding that significant protection was achieved with a dose of selenite of 1nM, whilst no significant protection was achieved with selenomethionine until a concentration of 50nM was present. With selenite maximal protection was achieved at a concentration of 10nM in all cell types whilst with selenomethionine maximal protection was achieved at concentrations of 100nM for keratinocytes and melanocytes.

Although both Se compounds displayed dose-dependant protection from UVB damage up to approximately 100nM, when the dose of Se was further increased protection appeared to diminish until at a dose of 1μM no significant protective effect on cell death was found. For selenite this loss in protection with increasing concentration of selenite appeared to be due to a direct toxic effect of selenite at high concentrations (Fig 2). For selenomethionine the mechanism leading to the loss of protection at higher doses is less clear since selenomethionine was not toxic at any of the concentrations used (Fig 2).

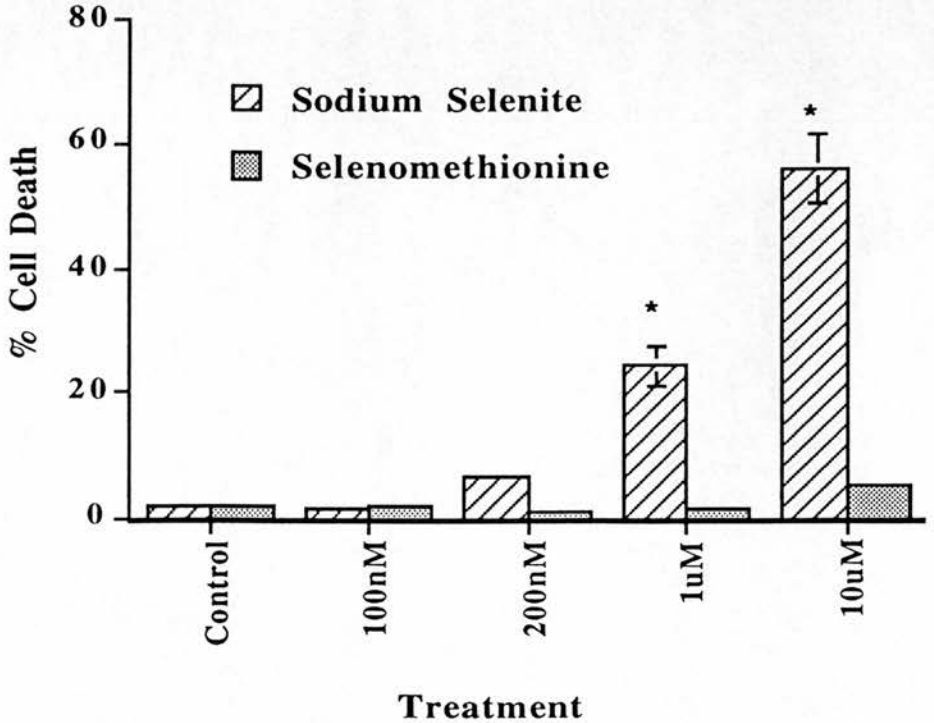

Figure 2 : The toxic effect of Se on the viability of primary human keratinocytes.

Cells were treated with sodium selenite or selenomethionine for 72 hours. Cells counts were performed using a heamocytometer and trypan blue exclusion. Control cells received no Se. Results are the means of the percentage cell death ± S.E.M, (n = 3). Significant difference from cells not treated with Se (= $p < 0.05$)*

Pre-incubation with Se was required to prevent UVB-induced damage. If Se was added immediately after UVB exposure little protection was achieved. This suggests that Se is acting through incorporation into selenoproteins rather than by a direct chemical action.

The family of GPXs and TR are clearly potential candidates for selenoproteins which may mediate the protective effects of Se since these enzymes are capable of detoxifying hydrogen peroxide, lipid hydroperoxides and phospholipid hydroperoxides which are produced during UVB exposure.

Primary keratinocytes, melanocytes and fibroblasts were found to have clearly different selenoprotein profiles (Fig. 3). Each cell type showed up to ten major selenoproteins (molecular mass ranging from approximately 10kDa to 60kDa), but many minor selenoproteins were also expressed. The expression of the 21kDa and the 60kDa selenoproteins showed the most diversity between cell types. These selenoproteins were identified by Western blotting as PHGPX and TR. We found that keratinocytes expressed much lower levels of TR and PHGPX than melanocytes and yet keratinocytes were found to be more resistant to UVB-induced cell death than melanocytes; typically a UVB dose of 720J/m^2 was required to produce 80% cell death in melanocytes whilst 960 J/m^2 was required to produce 80% cell death in keratinocytes. However, the susceptibility of a cell to UVB damage will depend on the total expression of a wide range of antioxidant enzymes and DNA repair mechanisms and not necessarily differences in the expression of a single specific selenoprotein.

Figure 3 : Selenoprotein expression by primary human skin cells.
Cells were labelled with [^{75}Se]-selenite [0.02 MBq] for 72 hours, the proteins were separated by SDS-PAGE and visualised by autoradiography.

We have shown that Se can protect both keratinocytes and melanocytes from UVB-induced damage and such protection can be achieved using very low concentrations of Se particularly when presented as sodium selenite. Intake of Se in the United Kingdom has fallen considerably over the past two decades such that, the average intake is now only about 40% of the recommended daily allowance (75µg/day) [10]. It is possible that a

contributing factor to the increase in the incidence of skin cancer of all types seen in the United Kingdom may, in part, be due to this decline in Se status.

ACKNOWLEDGEMENTS

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EXPRESSION OF SELENOPROTEINS BY SKIN CELLS AND PROTECTION FROM UVB INDUCED CELL DEATH BY SELENOMETHIONINE.

T S Rafferty, R C McKenzie, A F Howie and G J Beckett.
Departments of Dermatology and Clinical Biochemistry, University of Edinburgh, Lauriston Place Edinburgh EH3 9YW

Selenoproteins are thought to help prevent free radical induced damage. Although the functions of these selenoproteins are unknown, it has been shown in mice that selenium supplementation reduces ultraviolet-B radiation (UVB)-induced skin tumours.
We have characterised the expression of these selenoproteins by labelling skin cell cultures with ^{75}Se. We have found that primary keratinocytes, melanocytes and fibroblasts all express unique patterns of selenoproteins. Each cell type expresses between 7-9 species of selenoprotein. The major species are 56kd, 26kd, 21kd and 14kd. Keratinocyte cell lines had very similar patterns to the primary cells. As a role has been suggested for these proteins in protection from oxidative stress, we looked to see if selenium supplementation protected keratinocytes from UVB damage. We supplemented cells in culture with selenomethionine for 24h, gave a dose of UVB sufficient to kill 50% of the cells and determined viability after 48h. We found that 100nm selenomethionine gave the best protective effect, reducing the rate of cell death to 23%.

This is the first characterisation of selenoprotein expression in skin cells. These results suggest that selenium may protect cells from UV induced damage.

EXPRESSION OF SELENOPROTEINS BY SKIN CELLS AND PROTECTION FROM UVB-INDUCED CELL DEATH BY SELENIUM SUPPLEMENTATION.

T S Rafferty, R C McKenzie, A F Howie and G J Beckett.
Departments of Dermatology and Clinical Biochemistry, University of Edinburgh, Lauriston Place , Edinburgh EH3 9YW

Cells contain many different selenoproteins, however the functions of most of these selenoproteins are unknown. It has been shown in mice that selenium supplementation reduces ultraviolet-B radiation (UVB)-induced skin tumours. One possible mechanism to explain selenium's importance could be that some selenoproteins help prevent free radical-induced damage.

We have characterised the expression of skin cell selenoproteins by labelling cultures with 75Se followed by SDS polyacrylamide gel electrophoresis. We found that primary keratinocytes, melanocytes and fibroblasts all express unique patterns of selenoproteins. Each cell type expresses between 7-9 species of selenoprotein. The major species are 56kd, 26kd, 21kd and 14kd. Keratinocyte cell lines had very similar patterns to the primary cultures. As a role has been suggested for these proteins in protection from oxidative stress, we determined if selenium supplementation protected keratinocytes from UVB damage. We supplemented cells in culture with selenomethionine and sodium selenite for 24h, gave a dose of UVB sufficient to kill 50% of the cells (120J/m2/min) and determined viability after 48h. We found that 100nm selenomethionine and 50nm sodium selenite gave the best protective effect, reducing the rate of cell death to 19% and 27% respectively. We are repeating these survival experiments with UVA radiation, and determining the mechanism of this protective effect.

This is the first characterisation of selenoprotein expression in skin cells. These results suggest that selenium may protect cells from UV induced damage.

SELENIUM COMPOUNDS INHIBIT ULTRAVIOLET-B (UVB)-INDUCED KERATINOCYTE CYTOKINE PRODUCTION AND CELL DEATH BY APOPTOSIS.

T S Rafferty, G J Beckett*, J A A Hunter and R C M^cKenzie.
University Departments of Dermatology and *Clinical Biochemistry, The Royal Infirmary , Edinburgh EH3 9YW.

Ultraviolet radiation (UVR) can induce cell death by oxidative damage to cell lipids, proteins and DNA. UVR can also cause immunesuppression, by damage to Langerhans cells and induction of cytokines. It has been demonstrated that direct damage to DNA causes the release of immunesuppressive cytokines (O'Connor et al, J. Immunol 1996;157: 271-278) and that addition of selenium (Se) has been demonstrated to reduce the formation of DNA adducts in keratinocytes. We have previously reported that Se protects keratinocytes and melanocytes from UVB-induced cell death, presumably by acting as an antioxidant.

We sought to determine whether Se could prevent apoptotic cell death in keratinocytes, a indirect consequence of DNA damage. Apoptosis was maximal (30%) 16h after 680J/m^2 UVB. Pre-incubation with sodium selenite or selenomethione (10-200nM) for 24h decreased the level of apoptosis to 10% [$p<0.05$, $n=6$].

As DNA damage is implicated in cytokine production, we determined whether Se could prevent UVB-induced cytokine synthesis. Using RT-PCR, we looked at the effect of sodium selenite and selenomethionine on the production of mRNA for TNFα and IL-8. In human keratinocytes we found a two-fold induction of TNFα mRNA after 200J/ m^2 UVB. Pre-incubation with sodium selenite for 24h (1-10nM) decreased this induction by 50% and selenomethionine (50-200nM) by 60%. Four-fold induction of IL-8 was seen after 6h. Sodium selenite (50nm) decreased induction by 100% and selenomethionine (50-200nM) by 40%-85%. This effect was reproducible in mouse keratinocytes, where we found a 3-fold induction of TNFα 6h post UVB, sodium selenite (10-50nM) decreased this induction by 70%, and selenomethionine (10-50nM) by 50% [$p<0.05$, $n=3$].

This suggests that Se may protect the skin from UVB induced damage by reducing DNA damage and inflammatory cytokine production.

SELENIUM COMPOUNDS INHIBIT ULTRAVIOLET-B (UVB)-INDUCED KERATINOCYTE CYTOKINE PRODUCTION AND CELL DEATH BY APOPTOSIS. TS Rafferty, GJ Beckett*, JA Hunter and RC M^{c}Kenzie, Departments of Dermatology and *Clinical Biochemistry, The University of Edinburgh, Scotland

Ultraviolet radiation (UVR) can induce cell death by oxidative damage and cause immune suppression by damage to Langerhans cells. Others have demonstrated that damage to DNA causes the release of TNF-α and IL-10 (O'Connor et al, J. Immunol 1996;157: 271-278). Addition of selenium (Se) decreases the formation of DNA adducts in UVB-treated keratinocytes (KC) and protects KC and melanocytes from UVB-induced cell death. We sought to determine whether Se could prevent apoptotic cell death, an indirect consequence of DNA damage, in human KC. Apoptosis was maximal (30%) 16h after 680J/m^2 UVB. Pre-incubation with sodium selenite (SS) or selenomethionine (SM) (10-200nM) for 24h decreased the level of apoptosis to 10% [$p<0.05$, $n=6$]. As DNA damage is implicated in cytokine production, we determined whether Se could prevent UVB-induced cytokine synthesis. Using RT-PCR, we examined effects of SS and SM on the production of TNFα and IL-8 mRNA. In KC we found a two-fold induction of TNFα mRNA 6h after 200J/ m^2 UVB. Pre-incubation with SS for 24h (1-10nM) decreased this induction by 50% and SM (50-200nM) by 60%. Four-fold induction of IL-8 was seen after 6h. The SS (50nm) decreased induction by 100% and SM (50-200nM) by 40%-85%. This effect was reproducible in mouse KC, where we found a 3-fold induction of TNFα 6h post UVB, SS (10-50nM) decreased this induction by 70%, and SM (10-50nM) by 50% [$p<0.05$, $n=3$]. These data suggest that Se may protect the skin from UVB-induced damage by reducing DNA damage and inflammatory cytokine production.

SELENIUM PROTECTION AGAINST UVB-INDUCED APOPTOSIS OF EPIDERMAL CELLS MAY BE A CONSEQUENCE OF ITS ABILTY TO REDUCE p53 TRANSACTIVATION. N.J.Traynor, N.K.Gibbs, [1]T.S. Rafferty, [1]Y.C. Bisset, [2]J. Blaydes, [3]G.J. Beckett and [1]R.C. McKenzie. Photobiology Unit and [2]Molecular and Cellular Pathology, University of Dundee Medical School, DD1 9SY and [3]Dermatology and [4]Clinical Biochemistry, University of Edinburgh, EH8 9AG.

Ultraviolet radiation produces DNA damage including pyrimidine dimers (Pyr<>Pyr) and oxidised bases such as 8-hydroxy-guanine (8-OH-G). The tumour suppressor protein p53 is post-translationally activated by UVB radiation and acts as a transcription factor for downstream loci involved in cell cycle arrest (e.g. $p21^{WAF/CIP1}$) or apoptosis.(e.g. *bax*, *PIG2*). We have previously shown that low levels of selenomethionine (SM) protect cells from UVB-induced cell death and apoptosis (Rafferty *et al.* Biochem. J. 1998; **332**:231-236). We have also reported that SM protected against 8-OH-G formation but not from Pyr<>Pyr in the DNA of UVB irradiated cells. We now assess whether there is a connection between SM protection against UVB-induced apoptosis and p53 transactivation.

An amelanotic melanoma cell line expressing wild type p53 was stably transfected with the pRGCΔ*fos-lacZ* p53-dependent reporter construct (clone Arn8). Toxicity testing by the MTT assay revealed that SM doses of up to 100 μM for 24 h did not impair cell survival. After irradiation with TL12 broadband (~270-350nm) UVB lamps (0-30 mJ/cm2) the percentage of cells with transcriptionally active p53 (detected by X-gal substrate) increased dose-dependently up to 10 mJ/cm2, thereafter the response curve flattened, falling after 20 J/m2. Incubation with 50 uM SM for 24 h pre- and post-irradiation significantly diminished p53 transactivation by 1.5-1.9 fold across the UV dose range ($p<0.011$; n=5 independent experiments).

We now have evidence that SM protects against both UVB-induced apoptosis and p53 transactivation, which may suggest a causal link between the the two processes. The fact that SM protected against 8-OH-G, but not Pyr<>Pyr formation in epidermal cells, suggests that 8-OH-G may be an important UVB-induced DNA lesion in p53 transactivation and the subsequent apoptotic response.

unirradiated skin, measured using a reflectance instrument, was plotted against log UV dose. Sigmoidal curves fitted to these data (GraphPad PRISM software) were used to calculate for each lamp and at each time of measurement the UV doses required to cause a ΔE of 0·025 ($D_{0·025}$ – equivalent to just perceptible erythema) and ΔE of 0·1 ($D_{0·1}$ – equivalent to moderate erythema). Sufficient data to construct full dose–response curves for were obtained in 12 patients. A measure of curve steepness was made by calculating the $D_{0·1}$:$D_{0·025}$ ratio. The mean ratios at 24 h were 1·73 (95%CI 1·36–2·10) for TL-01 and 1·83 (1·52–2·14) for TL-12 (P = 0·64, paired t-test). At 72 h the mean ratios were 1·64 (1·34–1·95) for TL-01 and 1·73 (1·51–1·94) for TL-12 (P = 0·47). Persistence of erythema was measured by substituting the 24 h $D_{0·1}$ into the 72 h dose–response curve and calculating the ΔE that would have resulted from this dose. The mean calculated 72 h ΔE was 0·063 (0·043–0·084) for TL-01 and 0·068 (0·045–0·080) for TL-12 (P = 0·94).

Our results have shown no difference in the steepness of the dose–response or persistence of erythema induced by TL-01 or TL-12 lamps. Therefore, at least in terms of erythema, no evidence has been found for a difference in burning potential for the two lamp types.

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Effects of narrow band UVB (TL01) 311 nm on the non-lesional skin of psoriatics

B.D.DE SILVA, T.S.RAFFERTY,* M.NORVAL,* J.A.A.HUNTER* AND R.C.McKENZIE*

Department of Dermatology, the Royal Infirmary of Edinburgh, Edinburgh, U.K.

**Department of Medical and Radiological Sciences, University of Edinburgh, Edinburgh EH3 9YW, U.K.*

Phototherapy with narrow band UVB is an effective treatment for psoriasis. However, exposure to UVB causes erythema, DNA damage, immunosupression and lipid peroxidation We studied the effects of whole body irradiation with TL01 in patients with psoriasis.

18 subjects (8 male, 10 female, median age 41 (range 17–69) years) with psoriasis were randomly selected from those attending for TL01 treatment at the phototherapy department, The Royal Infirmary of Edinburgh, Scotland. Peripheral blood samples were collected at baseline and 48 h after the twelfth treatment for estimation of (1) natural killer cell activity by Chromium 51 (^{51}Cr) labelled tumour target cell assay and (2) lipid peroxidation by colourimetric assay. 4 mm punch biopsies were obtained under local anaesthetic from non-lesional buttock skin at baseline and after 12 treatments. These were immunostained with antibodies to p53 (indicative of DNA damage) and CD1a for Langerhans cells (LC). Statistical analysis was by Student's t-test.

All the patients had mild to moderate psoriasis with mean PASI score 6·5 (range 3·6–18·4). None were using topical tars or dithranol or had evidence of immunosuppressive disorder. There was no significant difference in the NK activity between the baseline and the post TL01 samples. Additionally lipid peroxidation was not significantly different in the post irradiation samples at 48 h. P53 immunostaining was increasedby TL01 exposure (unirradiated 4·34 ± 0·93 cells/mm of epidermis vs. irradiated 29·56 ± 7·73, P = 0·0089). There was a 26% reduction in the number of epidermal LC post TL01 (n = 11, unirradiated 17·03 ± 3·22 cells/mm of epidermis vs. irradiated 12·42 ± 2·99, P = 0·006).

TL01 caused DNA damage as shown by induction of p53 in the epidermis of these subjects with psoriasis. This has been demonstrated with broad band UVB and UVA but not as far as we are aware in human skin with TL01 exposure. In agreement with previous studies (El-Ghorr *et al.*). The effect of chronic low dose UVB radiation on Langerhans cells, sunburn cells, urocanic acid isomers, contact hypersensitivity and serum immunoglobulins in mice. Photochem. Photobiol., 1995, 62, 326–332.) we have shown a smaller reduction in epidermal LC post TL01 than with broadband UVB irradiation. However, no significant reduction in NK activity or systemic oxidative damage occurred in these subjects.

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Susceptibility of xeroderma pigmentosum keratinocytes from different complementation groups to ultraviolet B-induced apoptosis

C.PETIT-FRÈRE*, E.CAPULAS, J.E.LOWE, L.KOULU,† R.J.MARTTILA,‡ N.G.J.JASPERS,¶ P.H.CLINGEN,** M.H.L.GREEN†† AND C.F.ARLETT

MRC Cell Mutation Unit, University of Sussex, Brighton BN1 9RR, U.K.
**Division of Clinical Immunology, Karolinska Institute, S-141 86 Huddinge, Sweden*
†*Department of Dermatology and* ‡*Department of Neurology, University of Turku, FIN-20520, Turku, Finland*
¶*Department of Cell Biology and Genetics, Erasmus University, Rotterdam, PO Box 1738, NL-3000 DR, Rotterdam, the Netherlands*
***CRC Drug–DNA Interaction Research Group, Department of Oncology, UCL Medical School, 91 Riding House Street, London W1P 8BT, U.K.*
††*School of Pharmacological and Biomolecular Sciences, University of Brighton, Brighton BN2 4GJ, U.K.*

DNA damage induced by the ultraviolet component of sunlight plays an important role in the aetiology of skin cancer. Induction of cancer may be related not simply to the mutagenicity of sunlight, but to its ability to induce apoptosis, immunomodulation or cytokine release. If the endpoints other than mutagenesis are also mediated by DNA damage, then they should be altered in cases where DNA repair is defective. Patients from the UV repair deficiency syndrome xeroderma pigmentosum (XP) show enormously increased susceptibility to skin cancer, but other DNA repair syndromes are not necessarily cancer-prone. Resistance or susceptibility to apoptosis might affect the pool of UV-mutated skin cells available to progress to cancers in these syndromes. We have therefore measured UVB-induced apoptosis in keratinocytes grown out from fragments of skin biopsy from repair-deficient individuals. XP group A (3 biopsies) and XP group D (1 biopsy) keratinocytes were at least 6 times more sensitive than normal cells (2 biopsies) to UVB-induced apoptosis. XP variant keratinocytes (2 biopsies) showed intermediate susceptibility. XP group C keratinocytes (3 biopsies) proved heterogeneous, one sample showed high sensitivity to apoptosis, whereas two showed near normal susceptibility. Cockayne syndrome patients are defective in the repair of UV-induced DNA damage but not cancer-prone. The keratinocytes from 1 biopsy showed a similar susceptibility to that of XP groups A and D. Our work extends and partially confirms the work of Ljungman and Zhang[1] and suggests that relationships between repair deficiency, apoptosis and susceptibility to skin cancer are complex.

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Differential repair kinetics of ultraviolet-B radiation (UVB)-induced DNA damage in human keratinocytes (KC) and fibroblasts (FIBS) and protection from oxidative damage by selenium

T.S.RAFFERTY, M.H.L.GREEN,* J.E.LOWE,* C.ARLETT,* J.A.A.HUNTER, G.J.BECKETT† AND R.C.McKENZIE

EFFECTS OF SELENIUM DIETARY SUPPLEMENTS ON DNA DAMAGE AND IMMUNE FUNCTION IN NORMAL INDIVIDUALS AND PSORIASIS PATIENTS UNDERGOING PHOTOTHERAPY.

B. de Silva, [1]G.J.Beckett, [2]M. Norval, R.J. Langley, T.S.Rafferty, Y.C. Bissett, C.Walker, J.A.A. Hunter, R.C. McKenzie. Departments of Dermatology, [1]Clinical Biochemistry, and [2]Medical Microbiology, University of Edinburgh EH3 9YW.

Selenium (Se) can protect skin cells in culture from UVB-induced cell death by preventing DNA damage.
Similar studies have not yet been performed in human subjects. The present investigation examined the effects of supplementing the diet with daily capsules of 400 ug/day sodium selenite on the extent of DNA damage induced by irradiating lymphocytes in vitro with UVB, and on p53 expression and Langerhans cell (LC) numbers in the skin during phototherapy. Lymphocytes were purified from the blood of 6 normal subjects and irradiated with 40 J/m2 broadband UVB (Philips TL12). The DNA damage was assessed by the alkaline comet assay. The comet score [undamaged (1) to extensive damage (4)] in unirradiated cells before supplementation was 0.50 ± 0.06 which rose to 2.9 ± 0.00 in irradiated cells ($p<0.001$). After one week of selenite, the basal score was 0.95 ± 0.06 ($p<0.0001$) and the score after irradiation was 2.2 ± 0.00, significantly lower than the score before the supplementation ($p=0.0059$). The phototherapy group consisted of 10 psoriatic patients taking selenium supplementation and 9 taking a placebo, randomly allocated and assessed blindly. Biopsies were taken before the supplements were started and after 12 narrowband UVB exposures (Philips TL01) Epidermal LC numbers and cells expressing p53 were counted after immunostaining. Sunburn cells were assessed in H&E stained sections. The reduction in LCs as a result of phototherapy did not differ between the 2 groups, but keratinocytes expressing p53 increased 23-fold in the Se group compared with 6-fold in the placebo group ($p<0.05$). Sunburn cells were 4-fold fewer in the Se group following irradiation. Therefore Se supplementation modulated the effects of UVB on several parameters which are likely to influence immune function.

DIFFERENTIAL REPAIR KINETICS OF ULTRAVIOLET -B RADIATION (UVB)-INDUCED DNA DAMAGE IN HUMAN KERATINOCYTES (KC) AND FIBROBLASTS (FIBS) AND PROTECTION FROM OXIDATIVE DAMAGE BY SELENIUM.

T. Rafferty, M.H.L.Green[*] , J.E. Lowe [*], C. Arlett[*], JAA Hunter, G.J. Beckett[†], RC McKenzie. Departments of Dermatology and [†]Clinical Biochemistry, University of Edinburgh and [*]MRC Cell Mutation Unit, University of Sussex, UK.

Ultraviolet radiation is the most ubiquitous environmental genotoxic agent and the skin is the major target in humans for UVB-induced DNA damage. The trace element selenium (Se) is covalently incorporated into enzymes of the glutathione peroxidase and thioredoxin reductase families, which scavenge free radicals. We compared the ability of human KC and FIBS to repair cyclobutane dimers (CBD) and the effects of Se on DNA damage. Primary KC and FIBS were irradiated with 5 J/m^2 from FS20 lamps and subjected to T4 endonuclease treatment and comet assay 2h -72 h post UVB. Comet tail length increased from 43±8 μm to 82±200 μm. After 2h, 54% of this damage remained, 21% after 3h and only 10 % after 6h. In contrast, in FIBS comet length increased from 18±1 μm to 56±3 μm and this damage increased to 117% after 4h, dropping slowly with over 65% damage remaining after 24 h , 45% after 48 h and 30 % remaining after 72h (n=4). Treatment with up to 200 nM selenomethionine (SM) had no effect on CPD formation or rates of repair. In contrast, Se did protect against oxidative DNA damage measured by formation of Foramidopyrimidine glycosylase-sensitive (FaPy) sites, which are indicative of 8-hydroxydeoxyguanosine photoproduct formation. Irradiation of KC increased comet length from 54± 9 μm to 85± 17 μm. Pre-incubation for 24 h with 50 nM sodium selenite abolished UVB-induced increase in comet length ($P<0.001$). Pre-incubation with 10-200 nM SM dose-dependently protected KC from UVB-induced apoptosis 16 h after 600 J/m^2; 200 nM SM completely abolished UVB-induced apoptosis. Thus KC are more efficient than FIBS at repair of CBD and Se protects KC from UVB-induced oxidative damage and apoptosis, but not CPD formation.

SELENIUM INHIBITS ULTRAVIOLET RADIATION-INDUCED P53 ACTIVATION OF P53-RESPONSIVE ELEMENTS BUT DOES NOT PREVENT ACCUMULATION OF P53 PROTEIN IN EPIDERMAL CELLS.

N.J.Traynor, N.K.Gibbs, [1]T.S. Rafferty, [1]Y.C. Bisset, [2] J. Blaydes, [3]G.J. Beckett and [1]R.C. McKenzie,. Photobiology Unit and [2] Molecular and Cellular Pathology, University of Dundee Medical School, Dundee and Depts. of [1]Dermatology and [3] Clinical Biochemistry, University of Edinburgh.

Ultraviolet radiation damages DNA by several mechanisms including thymidine dimerisation and oxidative damage. The wild type p53 protein is a transcription factor which is activated in response to DNA damage; pre-existing p53 is stabilised and activated by post-translational modifications. The activated protein can then transactivate genes involved in cell cycle arrest in G1 or initiating apoptosis.(p21, bax, mdm2). Low levels of sodium selenite (SS) or selenomethionine (SM) protects cells from UVB-induced cell death and apoptosis (Rafferty et al. *Biochem. J.* 1998; 332:231-236). Since SM protect cells from oxidative DNA damage-but not thymidine dimers- we determined whether SM or SS affected p53 protein activation and accumulation.

An amelanotic melanoma cell line expressing wild type p53 was stably transfected with the pRGCΔfos-lacZ p53-dependent reporter construct (clone Arn8). After irradiation with FS12 broadband UVB lamps (0-30 mJ/cm^2) the percentage of cells with transcriptionally active p53 (detected by X-gal substrate) increased dose-dependently up to 25 mJ/cm^2. Incubation with 50 nM SM for 24 h post irradiation significantly diminished p53 activation by 1.9-1.5 fold across the UV dose range ($p=0.0014$ to 0.0101, $n=5$ independent experiments). Western blotting of Arn8 protein from parallel experiments showed that p53 abundance increased despite the presence of SM. We conclude that Se diminishes p53 activation but not protein accumulation; suggesting that oxidative DNA damage, not thymidine dimers, mediate p53 transactivation.